Genetic and chemical-molecular approach to study persistence in *Pseudomonas aeruginosa* to combat chronic infections

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Summary

Pseudomonas aeruginosa is an opportunistic pathogen that will initiate infection upon local or general impairment of the host immune system. As the number of people receiving immunosuppression and invasive treatments rises, *P. aeruginosa* infections are increasingly prevalent. *P. aeruginosa* is intrinsically resistant to many antibiotics but is also able to acquire additional resistance mechanisms, giving rise to strains unsusceptible to nearly all available antibiotics. However, even in the absence of detectable resistance, infections are often difficult to treat. A major factor responsible for treatment failure is the presence of persister cells, phenotypic variants of the wild type insensitive to the action of bactericidal agents. Upon withdrawal of the antibiotic, persisters revert to regular cells and regrow into a susceptible population, causing relapse of infection. They prolong the stay of the pathogen in the human body in the presence of antibiotics, leading to an increased probability of developing resistance. Targeting these persisters will greatly improve treatment.

Mechanisms underlying formation of *P. aeruginosa* persister cells are not fully understood. To obtain more insight into the mechanistic basis of persister formation in *P. aeruginosa*, a screening was conducted prior to this work to identify genes involved in fluoroquinolone tolerance in this pathogen. In a first part of this thesis, we follow up on this previous research by characterizing the newly identified gene named *dnpA* (de-*N*-acetylase involved in persistence). We demonstrate that *dnpA* is a *bona fide* persistence gene, since the persister level of the *dnpA* mutant after fluoroquinolone treatment is strongly reduced but increases upon overexpression of *dnpA* in the wild-type strain. Despite its genomic localization in the conserved lipopolysaccharide (LPS) core oligosaccharide biosynthesis gene cluster, no obvious role in LPS biosynthesis was demonstrated. A transcriptome analysis indicates that DnpA affects the expression of genes involved in surface-associated processes. In support of these results, we demonstrate that DnpA is inserted in the inner membrane. Additionally, our experiments reveal that correct transmembrane insertion of DnpA, but not membrane association *per se*, is needed to exert its effect on fluoroquinolone tolerance in *P. aeruginosa*.

It is becoming clear that several mechanisms are involved in the formation of persister cells, making rational design of an anti-persister therapy difficult. In a second part of this thesis, a new research line was initiated in which a top-down approach was used to identify compounds that, in combination with conventional antibiotics, reduce the persister fraction of *P. aeruginosa*. We identified three distinct compound families with anti-persister properties. Based on a structure activity relationship analysis, the two most promising molecules, named SPI001 and SPI002, each belonging to a different compound family, were selected for additional experiments. Both compounds are able to reduce the persister fraction of *P. aeruginosa* in combination with mechanistically different antibiotics. Importantly, they also show activity against other relevant Gram-negative and Gram-positive pathogens. Further characterization of SPI001 reveals biological activity against different pseudomonal clinical isolates as well as the ability to improve activity of additional classes of antibacterial agents. In addition, we provide evidence that this compound exerts its cidal effect by acting on the bacterial membrane. Our results show that SPI001 has the potential to be developed for use in an anti-persister therapy.

The work presented in this thesis contributes to the development of an anti-persister therapy, which prevents relapse of infection, and thus positively affects patients' outcome.

Samenvatting

Pseudomonas aeruginosa is een opportunistische pathogeen die infectie kan veroorzaken in personen waarin het immuunsysteem geheel of gedeeltelijk is verzwakt. Door een stijging in zowel het aantal personen met een onderdrukt immuunsysteem als patiënten die invasieve ingrepen ondergaan, zijn *P. aeruginosa* infecties meer en meer voorkomend. Deze pathogeen is intrinsiek resistent tegen vele verschillende klassen van antibiotica en verwerft bovendien ook gemakkelijk bijkomende resistentiemechanismen. Hierdoor zijn er reeds stammen beschreven die ongevoelig zijn voor bijna alle courant gebruikte antibacteriële middelen. Ook indien geen resistentie tegen het gebruikte antibioticum wordt vastgesteld zijn pseudomonale infecties vaak moeilijk te behandelen. Een belangrijke factor hiervoor verantwoordelijk is de aanwezigheid van persistorcellen, fenotypische varianten van het wildtype die ongevoelig zijn voor de bactericidale werking van antibiotica. Na daling van de antibioticumconcentratie op de plaats van infectie kunnen deze cellen ontwaken en groeien ze uit tot een gevoelige populatie, waardoor de infectie terug opflakkert. Op deze manier verlengen persistorcellen de tijd die de bacterie doorbrengt in de gastheer in aanwezigheid van antibiotica, wat de kans op ontwikkeling van resistentie verhoogt. Gericht inwerken op deze persistorcellen zal daarom de behandeling van infecties aanzienlijk verbeteren.

De mechanismen die verantwoordelijk zijn voor de vorming van persistorcellen in *P. aeruginosa* zijn nog niet volledig begrepen. Daarom werd eerder in onze onderzoeksgroep een screening uitgevoerd ter identificatie van genen betrokken in tolerantie tegen fluorchinolonen in *P. aeruginosa*. In het eerste deel van deze thesis wordt verder gewerkt op dit voorgaand onderzoek door een nieuw geïdentificeerd gen, dat *dnpA* werd genoemd (de-*N*-acetylase betrokken in persistentie), verder te karakteriseren. We tonen aan dat *dnpA* een *bona fide* persistentiegen is doordat overexpressie van het *dnpA* allel zorgt voor een stijging in het aantal persistorcellen terwijl een *dnpA* insertiemutant een lagere persistorftractie vertoont ten opzichte van het wildtype. Ondanks zijn genomische ligging in de geconserveerde lipopolysacharide (LPS) kern oligosacharide biosynthese cluster kon geen duidelijke rol worden vastgesteld voor DnpA in LPS synthese. Een transcriptoomanalyse impliceert dat

DnpA genen beïnvloedt die betrokken zijn in oppervlakte-gerelateerde processen. Deze resultaten worden verder ondersteund door onze observatie dat DnpA aanwezig is in de binnenmembraan van *P. aeruginosa*. Verder duiden onze experimenten erop dat correcte insertie van DnpA in de membraan, en niet zozeer membraanassociatie op zich, noodzakelijk is om zijn rol in fluorchinolonentolerantie te vervullen.

Het wordt steeds duidelijker dat verschillende overlappende mechanismen betrokken zijn in de vorming van persistorcellen, hetgeen de ontwikkeling van een doelwitgericht antipersistormiddel moeilijk maakt. In een tweede deel van deze thesis wordt een nieuwe onderzoekslijn opgestart met als doel kleine moleculen te identificeren die, in combinatie met conventionele antibiotica, de persistorftractie van *P. aeruginosa* doen dalen. We identificeerden drie verschillende componentfamilies die over zulke antipersistoractiviteit beschikken en op basis van een structuuractiviteitsanalyse werden de twee meest belovende componenten geselecteerd voor verdere testen. Deze componenten, SPI001 en SPI002, elk lid van een andere componentfamilie, zijn in staat om de persistorfractie significant te verlagen in combinatie met verschillende klassen van antibiotica. Bovendien vertonen ze ook anti-persistoractiviteit op andere klinisch relevante Gram-negatieve en positieve bacteriën. Nadere karakterisatie van SPI001 toont aan dat deze component ook actief is op klinische isolaten van *P. aeruginosa* en dat het de biologische activiteit van bijkomende klassen van antibacteriële middelen verbetert. Verdere experimenten wijzen erop dat SPI001 inwerkt op de bacteriële membraan. Onze resultaten tonen aan dat SPI001 het potentieel bezit voor verdere ontwikkeling tot een anti-persistortherapie.

Het werk in deze thesis draagt bij tot de ontwikkeling van een therapie gericht tegen persistorcellen. Op deze manier wordt de heropflakkering van infectie voorkomen hetgeen een gunstige invloed heeft op de gezondheid van de patiënt.

List of abbreviations

2-AA	2-aminoacetophenone
AHL	N-acylhomoserine lactone
ALT	antibiotic lock therapy
Am	amikacin
AME	aminoglycoside modifying enzyme
AMP	antimicrobial peptide
Ap	ampicillin
ATM	aztreonam
BCIP	5-Bromo-4-chloro-3-indolyl phosphate
BF8	(Z)-4-bromo-5-(bromomethylene)-3-methylfuran-2(5H)-one
C10	3-[4-(4-methoxyphenyl) piperazin-1-yl] piperidin-4-yl biphenyl-4-carboxylate
CAI	community-acquired infections
CAP	community-acquired pneumonia
Cb	carbenicillin
CD3	centre for drug design and discovery
c-di-GMP	cyclic di-GMP
CDI	contact dependent growth inhibition
Cf	ceftazidime
CF	cystic fibrosis
CFTR	cystic fibrosis transmembrane conductance regulator
CFU	colony forming units
cIAI	complicated intra-abdominal infections
CLSM	confocal laser scanning microscope
COPD	chronic obstructive pulmonary disease
Cp	ciprofloxacin
DMSO	dimethyl sulfoxide
DOM	defective *oprD* mutation

DOPC	1,2-dioleoyl-*sn*-glycero-3-phosphocholine
DOPE	1,2-dioleoyl-sn-glycero-3-phosphoethanolamine
DOPG	1,2-dioleoyl-sn-glycero-3-phospho-(1'-rac -glycerol)
eDNA	extracellular DNA
FDR	false discovery rate
Gm	gentamicin
HAI	healthcare-associated infections
HAP	hospital-acquired pneumonia
HPLC	high pressure liquid chromatography
IM	inner membrane
IPTG	isopropyl ß-D -1-thiogalactopyranoside
IPM	imipenem
Km	kanamycin
L-Ara4N	4-amino-4-deoxy-L-arabinose
LB	Luria-Bertani
LPS	lipopolysaccharide
MBIC	minimal biofilm inhibitory concentration
MBL	metallo-ß-lactamases
MEM	meropenem
MHB	Mueller Hinton broth
MIC	minimal inhibitory concentration
MMR	methyl-directed DNA mismatch repair
Of	ofloxacin
OM	outer membrane
ORF	open reading frames
PBS	phosphate buffered saline
ppGpp	guanosine 5'-diphosphate 3'-diphosphate
pppGpp	guanosine 5'-triphosphate 3'-diphosphate
QS	quorum sensing
QQ	quorum quenching
RBS	ribosome binding site
Rif	rifampicin
RND	resistance-nodulation-division

SAR	structure activity relationship
SCV	small colony variants
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
SEM	standard error of the mean
SRM	spontaneous resistant mutants
SUV	small unilamellar vesicles
TA	toxin-antitoxin
Tc	tetracycline
TSB	trypticase soy broth
UTI	urinary tract infection
VAP	ventilator-associated pneumonia
WT	wild type

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Chapter 1

The Gram-negative opportunistic pathogen *Pseudomonas aeruginosa*

1.1 Introduction

1.1.1 History of the genus *Pseudomonas*

Pseudomonas aeruginosa was first isolated by Gessard in 1882 from blue-green colored wound bandages (Gessard, 1984) and named *Bacillus pyocyaneus* (Silvestre & Betlloch, 1999). After more than a decade, Walter Migula proposed the genus *Pseudomonas*, which he defined as 'Cells with polar organs of motility. Formation of spores occurs in some species but it is rare' (Migula, 1894). As a type species, he proposed *Pseudomonas pyocyanea*, which was later renamed to *P. aeruginosa*. However, he never provided a clarification for his choice of the name '*Pseudomonas*'. Taxonomists interpreted the name as a derivation from the Greek words 'pseudes' (false) and 'monas' (unit). False would refer to the variability in morphology of these organisms (Silvestre & Betlloch, 1999). However, Palleroni suggested that Migula named *Pseudomonas* after a nanoflagellate called '*Monas*'. Cells of *Pseudomonas* resembled those of this nanoflagellate both in size and motility, which resulted in the name false '*Monas*' (Palleroni, 2010). '*aeruginosa*' is derived from the Latin name for copper rust, referring to the blue-green appearance of this bacterium (Silvestre & Betlloch, 1999; Palleroni, 2010). This color results from the production of the soluble pigment pyocyanine.

Because of the broad description by Migula, a large number of unrelated organisms were assigned to this genus. In 1984, over 100 species were classified as *Pseudomonas* as listed in Bergey's Manual of Systemic Bacteriology (Palleroni, 1984; Peix *et al.*, 2009). Later on, based on sequencing and comparison of 16S rRNA, 5 different rRNA groups were defined (Palleroni *et al.*, 1973). Currently, all members of Group II-V have been moved to other

genera such as *Burkholderia* and *Ralstonia* (Anzai *et al.*, 2000). rRNA Group I or *Pseudomonas sensu stricto*, contains the remaining *Pseudomonas* species with *P. aeruginosa* as type species. This group is divided into 8 different subgroups including *P. aeruginosa*, *P. fluorescens*, *P. putida*, *P. stutzeri*, *P. syringae*, *P. chlororaphis*, *P. pertucinogena* and *incertae sedis*.

1.1.2 Characteristics of *Pseudomonas aeruginosa*

P. aeruginosa is a non-spore forming, Gram-negative rod-shaped γ-Proteobacterium measuring 0.5 to 0.8 µm by 1.5 to 3.0 µm. Most strains contain a single polarly inserted flagellum which is used for motility. This bacterium grows aerobically but is capable of using nitrate or arginine as a final electron acceptor in the absence of oxygen, allowing anaerobic growth (Vander Wauven *et al.*, 1984; Haas *et al.*, 1992). Because of its remarkable adaptability to a variety of environments, *P. aeruginosa* is present in diverse ecological niches such as soil, water, plants and animals (Rahme *et al.*, 1995; Mahajan-Miklos *et al.*, 2000). *P. aeruginosa* synthesizes 2-aminoacetophenone (2-AA), producing a fruity, grape-like smell. This characteristic, along with the fact that this bacterium produces the blue colored soluble pigment pyocyanin, greatly helps in identifying an unknown colony as *P. aeruginosa*.

The complete genome of the widely used laboratory strain *P. aeruginosa* PAO1, a wound isolate (Holloway, 1955), was sequenced and published in 2000 (Stover *et al.*, 2000). The genome has a high G+C content (66.6 %) and consists of 6.3 million base pairs. The presence of 5,570 predicted open reading frames reflects the genetic complexity of the *P. aeruginosa* genome underlying the capability of this bacterium to grow in different ecological niches. At the moment, complete genome sequences of 30 additional *P. aeruginosa* strains are publicly available.[1]

P. aeruginosa can be part of the normal human microbial flora. For instance, this bacterium is present in the intestine of 10-15 % of healthy persons (White, 1971) and in about 2 % , *P. aeruginosa* is found in moist regions of the skin such as the external ear, armpits and anogenital regions (Hojyo-Tomoka *et al.*, 1973). However, *P. aeruginosa* is an opportunistic

[1] http://patricbrc.org/

pathogen that will initiate infection upon local or general impairment of the immune system (Bergan, 1981).

1.2 *Pseudomonas aeruginosa* as a human pathogen

P. aeruginosa causes community-acquired infections (CAI) but is especially known to cause healthcare-associated infections (HAI). CAI include skin infections, ear infections, keratitis and in rare cases pneumonia. In the latter case, patients almost always suffer from a general or local immune deficiency (Williams *et al.*, 2010). HAI caused by *P. aeruginosa* are often life-threatening and, since the number of people receiving immunosuppression and invasive treatments increases, are increasingly prevalent. Below, some examples of CAI and HAI are described.

1.2.1 Community-acquired *Pseudomonas* infections

1.2.1.1 Microbial keratitis

Microbial keratitis is a corneal infection that can result in severe vision loss due to scarring of the cornea. Several factors increase the incidence of microbial keratitis, with the most common ones being ocular surface disease, ocular trauma, contact lens wear, systemic diseases and ocular surgery (as reviewed in Stapleton & Carnt (2012)). The major preventable predisposing factors include ocular trauma and wearing contact lenses (Bourcier *et al.*, 2003; Keay *et al.*, 2006). The latter induces physiological changes to the corneal cells, lowering resistance to bacterial infection (as reviewed in Stapleton & Carnt (2012)).

P. aeruginosa is the most common cause of contact lens-associated microbial keratitis (Cheng *et al.*, 1999). This bacterium is often resistant to contact lens disinfectant fluid and grows as biofilms in lens storage materials. In addition, *P. aeruginosa* is capable of adhering to and colonizing lens materials during wear. Adherence to the lens surface is mediated by the cell-associated structures pili and flagella, but also depends on the hydrophobic character of the bacterial cell surface (Dutta *et al.*, 2012). Initiation and maintenance of infection relies on the production of extracellular and cell-associated virulence factors (Willcox, 2007). *Pseudomonas* produces several proteins, such as ExoS, ExoU, elastase, alkaline protease and

protease IV, that severely destruct the corneal cells. Importantly, excessive induction of host immune response greatly contributes to the destruction of the cornea.

Two phenotypic distinct corneal isolates, cytotoxic and invading strains respectively, have been detected (Fleiszig *et al.*, 1996). The phenotypic difference between those two strains depends on the presence of genes encoding the type III secreted virulence factors ExoS, ExoT, ExoU and ExoY. The cytotoxic strains typically lack the gene encoding ExoS (Fleiszig *et al.*, 1997) and are responsible for acute infection, hereby causing corneal edema (Cole *et al.*, 1998). The invasive strains do not produce ExoU (Fleiszig *et al.*, 1997) and enter the corneal cells, where they survive and replicate without killing the host cells (Fleiszig *et al.*, 1994; Fleiszig *et al.*, 1995). These invasive strains also cause severe keratitis resulting in corneal ulceration (Cole *et al.*, 1998).

1.2.1.2 Primary cutaneous infections

In healthy persons, *Pseudomonas* is capable of causing several cutaneous manifestations. The most important local predisposing factors are hyperhydration and destruction of the stratum corneum, the upper layer of the epidermis. This stratum corneum is the first line of defense against infection because of its dryness and the presence of sphingosines, lipids that possess antimicrobial properties (Silvestre & Betlloch, 1999). The prognosis of these skin infections in healthy persons is good, while in immunosuppressed people, the outcome is poor.

Common infections are folliculitis and the 'green nail syndrome'. **Folliculitis** is an infection and subsequent inflammation of hair follicles. Typically, in healthy persons, this disease is self-limiting and disappears within 7 to 10 days (Silvestre & Betlloch, 1999). The '**green nail syndrome**' or ungual infection results from the capability of *P. aeruginosa* to digest keratin (Lin *et al.*, 2009), the structural component of hair, nails and the outer layer of the epidermis. This infection is characterized by the green coloration of the nail fold which is reflected in the name. Most patients are cured within a few months, but in rare cases, removal of the affected nail is necessary (Silvestre & Betlloch, 1999). *P. aeruginosa* is also associated with **toeweb infections**. When feet are exposed to a hot and humid environment, the thick layer of stratum corneum is affected, facilitating the growth of *P. aeruginosa*.

1.2.1.3 Otitis externa

Acute otitis externa is an inflammation of the external ear canal, primarily caused by bacterial infection (Schaefer & Baugh, 2012). The most common predisposing factors are excessive moisture and trauma. As it is often caused by water left in the ear canal after swimming, this infection is also known as 'swimmer's ear'. *P. aeruginosa*, together with *Staphylococcus aureus* and *Staphylococcus epidermidis*, is the most common isolated pathogen in acute otitis externa (Roland & Stroman, 2002). Otitis externa sets off with itch, oedema, a sensation of fullness and, upon progression of the infection, with pain. This acute infection can become chronic upon inadequate treatment or when allergies or chronic dermatologic conditions are present (Ong & Chee, 2005; Schaefer & Baugh, 2012). In rare cases, *P. aeruginosa* invades the surrounding soft tissue and bone, which is called malignant otitis externa. This condition is potentially life-threatening and affects most frequently elderly, immunocompromised persons and diabetic patients (Hobson *et al.*, 2014).

1.2.2 Healthcare-associated infections

P. aeruginosa is a major cause of severe nosocomial infections (ECDC, 2013; Magill *et al.*, 2014), and is associated with a high percentage of morbidity compared to other bacteria (Harbarth *et al.*, 2002; Osmon *et al.*, 2004). In Europe, *P. aeruginosa* is the fourth most isolated microorganism in HAI, accounting for 8.9 % of all isolated bacteria. It is the predominant organism found in respiratory tract infections, the most common HAI. Furthermore, *P. aeruginosa* resides in the top five organisms isolated from surgical site (7.6 %), urinary tract (8.4 %), bloodstream (6.1 %) and gastrointestinal tract (2.5 %) infections (ECDC, 2013). In addition, burn wound patients are highly susceptible to acute or chronic superinfection with *P. aeruginosa* (Altoparlak *et al.*, 2004). Infections often initiate as localized lesions but can spread into deeper tissue, eventually resulting in life-threatening sepsis (Church *et al.*, 2006).

P. aeruginosa is capable of growing in a wide variety of environments, including specific niches within the hospital. It is capable of forming biofilms, multicellular structures of cells embedded in a self-produced extracellular matrix in which bacteria are more tolerant against the bactericidal action of antibacterial agents (Costerton *et al.*, 1999; Romling & Balsalobre, 2012). In this way, *P. aeruginosa* often persists within hospital equipment, forming a

reservoir of bacteria that may cause infection outbreaks. For example, *P. aeruginosa* biofilms in the hand hygiene sink drains of intensive care units served as a source for a severe outbreak of *P. aeruginosa* infection in the Toronto General Hospital (Toronto, Canada) (Hota *et al.*, 2009). In this specific case, 12 out of 36 infected patients died as a consequence of infection. Several other cases have been reported in which for example contaminated hand lotion (Becks & Lorenzoni, 1995), nebulizers (Cobben *et al.*, 1996), surface cleaning equipment (Engelhart *et al.*, 2002), medical equipment (Silva *et al.*, 2003), mouth swabs (Iversen *et al.*, 2007) and even contaminated bottled water (Eckmanns *et al.*, 2008) served as the source of infection.

P. aeruginosa is the dominant organism isolated in lung-associated infections (ECDC, 2013). Acute respiratory infections are mainly the result of trauma, for example after intubation or smoke inhalation. In many cases, *P. aeruginosa* can grow and form biofilms on the endotracheal tubes itself, forming a reservoir of bacteria that can initiate infection in the intubated patient (Adair *et al.*, 1999). On the other hand, chronic infections develop if the immune system of the patient is weakened, such as in patients receiving immunosuppression, immune deficient persons and the elderly. The classic example of chronic pseudomonal lung infections are the ones present in the cystic fibrosis lung (as discussed in the next section). Furthermore, chronic lung infections are also highly associated with people suffering from chronic bronchiectasis and obstructive pulmonary disease (COPD). COPD develops as the result of chronic inflammation of the lung, mostly because of chemicals present in cigarette smoke (Provinciali *et al.*, 2011). Approximately 4-15 % of COPD patients get infected by *P. aeruginosa*, with the signs of infection being either acute or chronic (Williams *et al.*, 2010).

1.2.3 Special case: pseudomonal infection of the cystic fibrosis lung

Cystic fibrosis (CF) is a recessive hereditary disorder caused by mutation of the CF transmembrane conductance regulator (CFTR), and is most common in North European populations. Over 1800 mutations in the CFTR have been identified[2] with the absence of phenylalanine at position 508 (ΔF508) accounting for almost 70 % of all mutations observed in North European and North American CF patients (Lyczak *et al.*, 2002; O'Sullivan & Freedman, 2009). Mutation of CFTR, a cAMP-dependent chloride ion transporter, results in malfunction or loss of epithelial transport of chloride. In addition, CFTR also has other

[2] Cystic Fibrosis Foundation. Cystic Fibrosis Foundation patient registry 2012 annual data report. 2013

regulatory roles such as inhibition of sodium transport (Ismailov *et al.*, 1996) and is involved in bicarbonate-chloride exchange (Smith & Welsh, 1992). This CFTR dysfunction leads to the accumulation of thick, dehydrated mucus in all exocrine glands, resulting in several clinical manifestations associated with CF.

The majority of CF patients is born with or develop pancreatic insufficiency within the first year of life, resulting in vitamin deficiency and malnutrition. Currently, the life expectancy of CF patients is over 40 years[3], but at the time CF was first recognized, they only survived a few months because of malnutrition (Anderson, 1938). Nowadays, patients receive pancreatic enzyme replacement therapy (Borowitz *et al.*, 2002). As the patient ages, the pancreas undergoes autolysis, eventually leading to insulin insufficiency and carbohydrate intolerance (Elder *et al.*, 2007). In addition, the thickened intestinal secretions lead to intestinal obstruction and blocked bile ducts. In male CF patients, mutation of CFTR results in abnormalities of the reproductive system. They lack the vas deferens, resulting in infertility (Quinzii & Castellani, 2000). Presently, the most life-threatening clinical manifestation of CF patients is situated at the respiratory system. Because of the dehydrated and thickened airway surface liquid present in the CF lung, mucociliary clearance is impaired (Hartl *et al.*, 2012), facilitating bacterial colonization. Typically, soon after birth, lungs are colonized with *S. aureus* and/or *Haemophilus influenza*. Infection by these bacteria damages the epithelial cells, facilitating the attachment of *P. aeruginosa*, which in time becomes the dominant bacterial species present in the CF lung (Folkesson *et al.*, 2012). *P. aeruginosa* adapts to the CF lung environment by switching to a mucoid phenotype, reducing the expression of virulence factors and by adaptation of metabolic pathways to the lung environment. The concentrated airway mucus also likely promotes formation of microcolonies and biofilms (Matsui *et al.*, 2006). In this way, infections caused by *P. aeruginosa* persist and become chronic (Hogardt & Heesemann, 2013). In addition to the continuous damage caused by the bacteria, the presence of *P. aeruginosa* leads to immune-mediated inflammation, which further damages the lung tissue resulting in decreased lung function. It was shown that patients who remain clear of *P. aeruginosa* have a significantly better survival rate (Konstan *et al.*, 2007). Consequently, early detection and eradication of infections is highly recommended.

[3] Cystic Fibrosis Foundation

1.3 Current treatment problems

Nosocomial infections with *P. aeruginosa* are often caused by strains resistant to one or more classes of antibiotics (ECDC, 2013). The low number of potent new anti-pseudomonal drugs in late stages of clinical development (see Section 1.4.1) makes effective treatment very hard and, in some cases, nearly impossible. Some infections are difficult to eradicate despite the absence of detectable resistance against the used antibiotic of the isolated strains (Mulcahy *et al.*, 2010). The latter may be explained by the formation of biofilms, in which cells are more tolerant to antibiotics, and the presence of persister cells. Persisters are suspected to be the major culprit of relapse of infection, giving rise to persistent and even chronic infections. Lingering, untreatable infections caused by multi-drug resistant strains prolong the hospital stay of patients and significantly contribute to morbidity and mortality. It is estimated that in 2007, HAI caused by resistant strains resulted in 2.5 million extra hospital days in the European Union, Iceland and Norway and in addition, were responsible for 25,000 death cases (ECDC, 2009). Below, the mechanisms of antibiotic resistance and factors underlying the difficult treatment of infections caused by non-resistant strains are discussed.

1.3.1 Antibiotic resistance

P. aeruginosa is intrinsically resistant to a number of antibiotics and has the remarkable ability to quickly and easily acquire additional resistance mechanisms, giving rise to strains resistant to nearly all antibiotics. As new anti-pseudomonal drugs are not expected to be introduced on the market soon (Bassetti *et al.*, 2013; Pendleton *et al.*, 2013), infections may become untreatable. Furthermore, the absence of good treatment options of bacterial infections, including those caused by *Pseudomonas*, makes important medical interventions such as chemotherapy and invasive surgery impossible, since they rely on protection of the patient offered by antibiotics. Currently, old drugs such as polymyxins, although reported as nephro- and neurotoxic (Ryan *et al.*, 1969; Koch-Weser *et al.*, 1970), are increasingly used as a last resort in treatment of critically ill patients (Nation & Li, 2009; Boisson *et al.*, 2013). In the following paragraphs, mechanisms underlying intrinsic resistance and strategies that *P. aeruginosa* uses to obtain resistance against additional antibiotics are described.

1.3.1.1 Intrinsic antibiotic resistance

P. aeruginosa is intrinsically resistant to a number of antibiotics, resulting from an interplay between different mechanisms, encoded by the genetic make-up of this bacterium (Breidenstein *et al.*, 2011). The low overall membrane permeability reduces the entrance rate of antibiotics in the bacterial cell. Antibiotics that manage to cross the outer membrane and enter the bacterial cell are extruded by the action of efflux pumps or are degraded by chromosomally encoded inactivating enzymes.

Outer membrane impermeability

The outer membrane of Gram-negatives is highly impermeable for large hydrophobic and hydrophilic molecules. Even diffusion of small molecules is restricted and uptake of nutrients occurs via specific porins (Nikaido, 2003). Small hydrophilic antibiotics such as the ß-lactams and quinolones usually diffuse across the outer membrane, mostly through small aspecific porin channels (Nikaido, 2003; Delcour, 2009). The major porin protein in *P. aeruginosa* is OprF (Hancock *et al.*, 1979) forming large pores in the outer membrane (Bellido *et al.*, 1992). Despite the fact that the number of OprF proteins is similar to that of the major *Escherichia coli* porin protein, the *P. aeruginosa* outer membrane is 100 times less permeable for hydrophilic solutes as compared to *E. coli* (Yoshimura & Nikaido, 1982). This reduced rate of antibiotic influx is explained by the observation that *P. aeruginosa* contains a very limited number of open channel porins (Nikaido, 2003). The small size of the other porins such as OprD and OprB further prevents diffusion of antibiotics across the outer membrane (Nikaido, 2003; Breidenstein *et al.*, 2011). Entrance of lipophilic molecules via diffusion through the outer membrane is significantly reduced by the presence of the lipopolysaccharide (LPS) leaflet (Plesiat & Nikaido, 1992).

Efflux pumps

Antibiotics that manage to cross the bacterial membranes are extruded by the action of efflux pumps. In Gram-negative bacteria, efflux pumps mainly belong to the resistance-nodulation-division (RND) family which operates as a tripartite system containing a periplasmic membrane fusion protein, an outer and an inner membrane protein (Poole, 2004). The genome

of *P. aeruginosa* encodes 12 RND systems, but only seven of them have been characterized. These include MexAB-OprM (Poole *et al.*, 1993; Li *et al.*, 1995), MexCD-OprJ (Poole *et al.*, 1996), MexEF-OprN (Kohler *et al.*, 1997), MexGHI-OprD (Aendekerk *et al.*, 2002; Sekiya *et al.*, 2003), MexJK-OprM/OpmH (Chuanchuen *et al.*, 2002; Chuanchuen *et al.*, 2005), MexVW-OprM (Li *et al.*, 2003) and MexXY-OprM (Mine *et al.*, 1999). MexGHI-OprD contains an additional small integral membrane protein MexG, of which the function is unknown (Aendekerk *et al.*, 2002). The most important pumps in terms of antibiotic resistance are MexAB-OprM, MexCD-OprJ, MexEF-OprM and MexXY-OprM, which have been identified in clinical isolates and are known to export clinically relevant antibiotics. All efflux pumps export a preferential set of substrates with MexAB-OprM showing the broadest substrate range, exporting antibiotics (ß-lactams, aminoglycosides, fluoroquinolones, macrolides, tetracyclines, chloramphenicol, novobiocin, lincosamides, glycylcyclines, trimethoprim) and non-antibiotic compounds (crystal violet, acriflavin, ethidium bromide, sodium dodecyl sulfate and triclosan). For an overview of the substrates of each independent efflux pump see for instance Kumar & Schweizer (2005) or Poole (2007). MexAB-OprM, MexXY-OprM and MexHI-OprM/H are involved in intrinsic resistance of *P. aeruginosa*, others such as MexCD-OprJ and MexEF-OprN are not expressed during growth under normal laboratory conditions.

Chromosomally encoded ß-lactamases

The chromosome of *P. aeruginosa* contains two genes that code for ß-lactamases, *ampC* and *poxB*. AmpC is a member of the class C ß-lactamases, and is linked to the intrinsic resistance of *P. aeruginosa* against ß-lactams (Lodge *et al.*, 1990; Poole, 2011). PoxB belongs to the ß-lactamases class D (Kong *et al.*, 2005), and its activity is only detected in strains lacking AmpC activity (Poole, 2011). AmpC is usually present in low levels but production may increase upon induction by the presence of a number of ß-lactams (Livermore & Yang, 1987). Interestingly, although they serve as a substrate for AmpC, monobactams, piperacillin and a number of cephalosporins do not induce production of this ß-lactamase (Poole, 2011). Therefore, resistance depends on mutational derepression of AmpC. Interestingly, AmpC activity is not inhibited by clinically used ß-lactamase-inhibitors such as clavulanic acid, sulbactam and tazobactam (Nordmann & Guibert, 1998). The presence of extended spectrum AmpC variants has been reported among clinical isolates (Rodriguez *et al.*, 2009).

1.3.1.2 Acquired antibiotic resistance

P. aeruginosa has a remarkable capacity to acquire additional resistance mechanisms to multiple classes of antibiotics (as reviewed by Strateva & Yordanov (2009) and Breidenstein *et al.* (2011)). To this end, it uses two different mechanisms, mutational resistance and horizontal gene transfer, which are described below.

Mutational resistance

Additional resistance can be acquired by mutation of antibiotic target genes. This is most common for quinolone resistance, where specific point mutations in genes encoding DNA gyrase or topoisomerase IV confer resistance to this class of antibiotics (Jacoby, 2005). The main target of quinolones in Gram-negatives is DNA gyrase, but high levels of resistance are usually obtained when mutations in both enzymes are present. These point mutations occur in the active site of these enzymes, also called the quinolone-resistant-determinative region (Yoshida *et al.*, 1990; Yoshida *et al.*, 1991), and lead to lower binding affinity for the quinolones.

Mutational resistance can also be obtained by mutations in non-target genes. Typically, these mutations enhance the intrinsic resistance already present in the bacterium. For example, partial derepression of the chromosomally encoded ß-lactamase AmpC is achieved by mutation of its negative regulator *ampD* (Langaee *et al.*, 2000). Constitutive hyperexpression of *ampC* is realized when additional mutations occur in the two AmpD homologues AmpDh2 and AmpDh3 (Juan *et al.*, 2006). Another example is provided by mutational inactivation of efflux pump repressors. As a consequence, genes encoding efflux pumps are overexpressed leading to increased resistance towards several antibiotic classes. For instance, mutation of *mexZ,* which is the most frequently reported mutation identified in CF clinical isolates (Smith *et al.*, 2006), results in the overexpression of the MexXY-OprM pump, conferring resistance to aminoglycosides, fluoroquinolones and the cephalosporin cefepime (Muller *et al.*, 2011). Lastly, carbapenem resistance is obtained when mutational alteration or loss of OprD occurs, accompanied with expression of AmpC (Livermore, 1992).

Horizontal gene transfer

P. aeruginosa can acquire DNA elements containing resistance genes through horizontal gene transfer by means of conjugation, transformation or transduction. This mechanism mainly affects aminoglycoside and ß-lactam resistance. Some examples are listed below.

The major mechanisms by which *P. aeruginosa* acquires additional resistance against ß-lactams is by horizontal transfer of plasmids encoding ß-lactamases. Based on their nucleotide and amino acid sequence, ß-lactamases are classified into four different classes, A-D. Members of all four classes have been identified in *P. aeruginosa* strains (extensively reviewed by Strateva & Yordanov (2009)).

Aminoglycoside resistance genes obtained by horizontal gene transfer encode aminoglycoside modifying enzymes (AMEs) or 16S rRNA methylases. AMEs include aminoglycoside phosphoryltransferases, aminoglycoside adenylyltransferases and aminoglycoside acetyltransferases that transfer a phosphate, adenyl or acetyl group, respectively, to the aminoglycoside antibiotic (as reviewed in Ramirez & Tolmasky (2010)). 16S rRNA methylases methylate, as the name suggests, 16S rRNA hereby interfering with the binding of aminoglycosides to the 30S ribosomal unit. This results in high-level resistance to the clinically used antibiotics amikacin, gentamicin and tobramycin (as reviewed in Doi & Arakawa (2007)). Examples of such enzymes are RmtA, RmtB, RmtD and ArmA.

Plasmid-encoded genes can also contribute to quinolone tolerance. For instance, the *qnr* genes encode proteins that bind DNA gyrase and topoisomerase IV hereby lowering the amount of enzyme that can bind to DNA and preventing quinolones to enter the cleavage complexes formed by these enzymes (Tran *et al.*, 2005a; Tran *et al.*, 2005b). The aac(6')-Ib-cr protein is capable of acetylating fluoroquinolones, thus decreasing drug activity (Robicsek *et al.*, 2006). Lastly, plasmids may contain genes encoding additional efflux pumps such as OqxAB, QepA1, and QepA2 (Yamane *et al.*, 2007; Cattoir *et al.*, 2008).

1.3.2 Recurrent infections

Even in the absence of genome-encoded resistance against the used antibiotic, treatment of *P. aeruginosa* infections remains challenging. Incomplete eradication of acute *P. aeruginosa* populations leads to relapse of infection, and, upon adaptation of *P. aeruginosa* to the host

environment, infections may become chronic. Furthermore, upon continuous presence of this bacterium in the host environment, the probability of acquiring additional resistance mechanisms increases, hereby contributing to the development of untreatable multi-drug resistant strains (Cohen *et al.*, 2013).

Several factors may contribute to the incomplete eradication of acute *P. aeruginosa* infections. First of all, *P. aeruginosa* is capable of altering its gene expression and/or protein expression upon sensing antibiotic stress. This phenomenon, known as adaptive resistance, is transient and the bacterium reverts back to its initial antibiotic sensitive state when the antibiotic disappears (Fernandez *et al.*, 2011). Secondly, the presence of a small fraction of persister cells, phenotypic variants of the wild type insensitive to the action of bactericidal agents, prevents total clearance of the bacterial population upon antibiotic treatment (Spoering & Lewis, 2001). As *P. aeruginosa* primarily infects immunocompromised persons, the immune system is not capable of removing this small remaining fraction of cells, hereby providing a reservoir of bacteria capable of causing relapse of infection.

Chronic infections are often associated with biofilm formation, multicellular structures of bacteria embedded in a self-produced extracellular matrix (Costerton *et al.*, 1999). Bacterial cells within biofilms are more tolerant to antibiotic treatment compared to free-living cells, making treatment more difficult. Examples of *P. aeruginosa* biofilm-associated infections include CF lung infection, chronic wound infection, catheter-associated urinary tract infection, chronic otitis media and contact lens-related keratitis (Romling & Balsalobre, 2012).

1.3.2.1 Adaptive resistance

Adaptive resistance is a transient phenomenon in which bacteria alter their gene expression and/or protein synthesis in response to changing environmental conditions. When the environmental trigger disappears, bacteria usually revert back to their initial antibiotic susceptible state. This temporary state allows the bacterium to cope with, for instance, a sudden increase in antibiotic concentration (Fernandez *et al.*, 2011).

An example of adaptive antibiotic resistance is the response of bacteria to cationic peptides such as polymyxins. The presence of this antibiotic induces the expression of the LPS

modification operon *arnBCADTEF*-PA3559 (Fernandez *et al.*, 2010). This gene cluster is responsible for adding 4-amino-4-deoxy-L-arabinose to the phosphate group of lipid A, thereby increasing tolerance to cationic antimicrobial peptides (Moskowitz *et al.*, 2004). Additional examples include the induction of the chromosomally encoded ß-lactamase AmpC by the presence of ß-lactams or upregulation of the genes encoding the MexXY-OprM efflux pump in the presence of aminoglycosides. In addition, adaptive resistance to antibiotics is not always triggered by the presence of antibiotics. For instance, social behavior such as the formation of biofilms or swarming induces the expression of genes involved in antibiotic resistance (as reviewed in Fernandez *et al.* (2011)).

1.3.2.2 Biofilm tolerance

Several factors contribute to the increased tolerance of biofilm-associated bacteria compared to planktonic cultures. A number of independent studies showed that penetration of aminoglycosides in biofilms was slower compared to that of fluoroquinolones (Hoyle *et al.*, 1992; Yasuda *et al.*, 1993; Kumon *et al.*, 1994; Suci *et al.*, 1994; Vrany *et al.*, 1997). Together with the observations that aminoglycosides were bound by alginate (Nichols *et al.*, 1988; Hatch & Schiller, 1998), it was hypothesized that the negatively charged biofilm matrix binds the positively charged aminoglycosides, slowing down diffusion of these antibiotics within the biofilm. A study carried out by Walters *et al.* (2003) indicated that the slower penetration of aminoglycosides does not explain the observed antibiotic tolerance of biofilms. Instead, limitation of oxygen, which results in lower metabolic activity of cells deeper in the biofilm, appeared to be an important factor contributing to biofilm tolerance. This is confirmed by the observation of the presence of different cell populations within the biofilm (Werner *et al.*, 2004). Due to diffusion, cells residing close to the substratum receive less oxygen and nutrients, resulting in a lower metabolism compared to cells located at the biofilm surface. Antibiotic tolerance of the biofilm was attributed to the presence of these metabolically-quiescent, slow-growing cells which are less susceptible to the action of antibiotics. However, this mechanism cannot explain the higher tolerance of biofilms to quinolones, since this class of antibiotics is capable of killing non-growing planktonic cells (Brooun *et al.*, 2000). Another mechanism that is believed to contribute to biofilm tolerance is the induction of adaptive resistance mechanisms. For instance, biofilm mode of growth induces expression of genes involved in synthesis of periplasmic glucans that bind

aminoglycosides (Mah *et al.*, 2003). In addition, genes PA1874-1877, encoding a biofilm-specific efflux system, are induced, resulting in increased resistance to tobramycin, gentamicin and ofloxacin (Zhang & Mah, 2008). At the beginning of the 21st century, it was observed that biofilms, just like planktonic cultures, contain a small fraction of persister cells (Spoering & Lewis, 2001). The presence of these antibiotic-insensitive cells is currently seen as a major culprit for the multi-drug tolerant character of a biofilm (see Chapter 2).

In addition to the high tolerance against antibiotics, the mutation frequency and horizontal gene transfer in biofilms is higher compared to planktonic cultures (Molin & Tolker-Nielsen, 2003; Driffield *et al.*, 2008), hereby increasing the rate of resistance development.

1.3.2.3 Adaptation of Pseudomonas to the host environment

Upon initial infection of the CF airway, *P. aeruginosa* undergoes several adaptations that ultimately result in chronic colonization of the CF lung (Hogardt & Heesemann, 2013). During this adaptation process, *P. aeruginosa* hypermutator phenotypes emerge, creating multiple subclonal variants of the initial colonizing strain, thereby enhancing long-term survival within changing environmental conditions. Changes are triggered by the lung environment and occur at the morphological, genetic and physiological level. The resulting chronic *P. aeruginosa* isolates are less inflammatory and cytotoxic compared to the initial colonizing isolates.

Hypermutator phenotype

Within the chronically infected CF lung, *P. aeruginosa* hypermutators are extremely prevalent (Oliver *et al.*, 2000). These strains possess an increased mutation rate due to defects in DNA repair systems. *P. aeruginosa* possesses different DNA repair systems that are involved in replacement of wrongly inserted nucleotides into the DNA and in prevention of recombination events between non-identical DNA sequences (Jolivet-Gougeon *et al.*, 2011). Members of the methyl-directed DNA mismatch repair (MMR) are most frequently mutated, while mutations in a second DNA repair system, 8-oxo-2-deoxyguanosine, are less reported among isolates from the CF lung. The MMR system involves the *mutS*, *mutL* and *uvrD* genes, with *mutS* the most commonly affected gene among CF isolates (Ciofu *et al.*, 2010). The

presence of hypermutators is correlated with a poorer lung function in CF patients (Ferroni *et al.*, 2009). This might be explained by the fact that the presence of hypermutators is correlated to an increased resistance rate (as reviewed by Oliver & Mena (2010)). As a consequence, isolates from the CF lung are more resistant compared to isolates obtained from other sources (Henwood *et al.*, 2001). More recently, evidence was provided that hypermutators also affect the genetic adaptation of *P. aeruginosa* to the CF lung, resulting in more recalcitrant strains, negatively affecting the clinical outcome of CF patients (Oliver & Mena, 2010).

Adaptations

To establish a chronic infection in the CF lung, *P. aeruginosa* undergoes changes at the **morphological level**. Chronic infections are marked by the presence of mucoid strains that overproduce the exopolysaccharide alginate. These isolates are more difficult to eradicate and are associated with increased tissue damage leading to deterioration of lung function (Hoffmann *et al.*, 2005). Overproduction of alginate is often the result of *mucA* inactivation as a response to oxidative stress (Mathee *et al.*, 1999). This leads to overexpression of AlgT/AlgU, an alternative sigma factor that induces expression of AlgD and several regulatory proteins, resulting in increased alginate levels (Ramsey & Wozniak, 2005). Mucoid isolates are better protected against stresses from the CF lung environment such as reactive oxygen species, antibiotics, antibodies and phagocytosis. A second phenotype detected in CF lungs is the occurrence of small colony variants (SCV) of which the presence in CF airways correlates with poor lung function and antibiotic therapy (Haussler *et al.*, 1999; Haussler, 2004). These variants display several characteristics that differ from their parental phenotype including a slower growth rate, resistance to antibiotics, increased biofilm formation and reduced motility. In the absence of antibiotics, they revert back to the wild type morphotype (Proctor *et al.*, 2006). Evidence is present that intracellular levels of the second messenger cyclic di-GMP (c-di-GMP) (Hengge, 2009) play a role in switching to the SCV phenotype (Starkey *et al.*, 2009). Specifically, it was shown that the tripartite signaling system YfiBNR, which controls c-di-GMP levels in *P. aeruginosa*, is involved in the generation of SCV. In response to yet unknown signals, YfiN produces c-di-GMP which targets exopolysaccharide synthesis, thereby leading to generation of SCV (Malone *et al.*, 2010). A follow-up study showed that several causal adaptive mutations in *P. aeruginosa* SCV were mapped to the *yfiBNR* locus. Some isolates harboured both activating and inactivating mutations, pointing to

the alternation between favorable and non-favorable lung environments for SCV throughout the course of infection (Malone *et al.*, 2012).

Adaptation to the CF lung is marked by changes in the expression of **virulence factors**. Several of them are switched off or produced in lesser amount to reduce recognition by the immune system and to lower energy costs associated with maintaining production of these factors (Hoboth *et al.*, 2009). For instance, chronic CF isolates lack the cell-associated virulence factors pili and flagella, mainly resulting from mutations of the *rpoN* gene (Mahenthiralingam *et al.*, 1994). Reduction in expression of several other virulence factors results from mutational inactivation of the quorum sensing (QS) transcriptional regulator *lasR*, a mutation very commonly found among chronic CF airway isolates (Smith *et al.*, 2006). Recently, it was shown that exposure of a *mutS*-deficient strains to 2-AA, a QS regulated molecule, caused accumulation of *lasR* mutations (Kesarwani *et al.*, 2011). In addition, cytotoxicity is severely reduced in chronic CF isolates, resulting from the absence of expression of the type III secretion system (Jain *et al.*, 2004).

In the CF lung, *P. aeruginosa* experiences various nutritional stresses and encounters a significantly lower oxygen tension, which results in **metabolic adaptations**. Several studies show that *P. aeruginosa* uses amino acids and fatty acids and TCA intermediates which are present in the nutrient-rich CF sputum, to support its growth in this environment (as reviewed by Hogardt & Heeseman (2013)). Analysis of sequential isogenic isolates obtained during chronic pulmonary infections further supported these results and, in addition, indicated that *P. aeruginosa* adapts to the micro-aerobic niches present in the CF lung (Hoboth *et al.*, 2009; Oberhardt *et al.*, 2010). Increased expression over time was observed for genes with a role in amino acid biosynthesis and metabolism, central carbon and energy metabolism and fatty acid and phospholipid metabolism. Decreased expression was observed for genes encoding cytochrome oxidases, generally expressed under aerobic conditions, and genes necessary for catabolism of aromatic compounds. Furthermore, expression of anaerobic and micro-aerobic respiration and the anaerobic arginine deaminase pathway was detected, supporting adaptation to the micro-aerobic niches present in the CF airway.

1.4 Novel treatment strategies

Incomplete eradication of acute infections leads to relapse and eventually, the infection can become chronic. Such infections not only seriously hampers the patients' outcome but also increase the cost associated with the prolonged hospital stay (ECDC, 2009). Old antibiotics like polymyxins, despite their toxic side effects, are currently used as a last option to treat critically ill patients (Nation & Li, 2009; Boisson *et al.*, 2013). A recent report of the World Health Organization on the prevalence of antimicrobial resistance revealed that a high level of resistance is found in all regions of the world[4], underlining the importance of finding new antibacterial agents.

Resistance develops soon after the introduction of a new antibiotic on the market. In case of daptomycin, resistance was observed within one year (Dolgin, 2010). Therefore, in addition to new antibiotics, novel treatment strategies are needed that slow down the rate of resistance development and completely eliminate the bacterial population, preventing an infection from becoming chronic. A good strategy entails combining different antibiotics or using a combination of an antibiotic with a compound targeting non-essential processes such as virulence, biofilms or persister cells. In addition, treatment strategies such as immunization, immunotherapy and phage therapy could be an alternative to deal with *P. aeruginosa* infections.

1.4.1 Novel antibiotics

To efficiently develop new antibacterials, reliable screening platforms need to be established (Lewis, 2013). These should focus on preventing rediscovery of existing antibiotic classes, incorporate rules describing the penetration of compounds through the bacterial cell envelope and explore new natural sources. Below, a brief history of antibiotic discovery is given, followed by two paragraphs that discuss possible new sources and approaches to increase discovery of new antibiotic classes. Finally, an overview is provided of anti-pseudomonal drugs currently under clinical development.

[4] WHO (2014) Antimicrobial resistance: global report on surveillance

1.4.1.1 History

Between 1940 and 1962, about 20 different antibiotic classes were introduced on the market, with most of them discovered by screening soil-derived actinomycetes. Resistance to these antibiotics did emerge, but given the high number of newly identified antibiotics, it was believed that the battle against infectious diseases was won. However, since the 1960s, only a few new classes of antibiotics have been approved, and problematically, they lack activity against Gram-negative bacteria (as reviewed by Coates *et al.* (2011) and Lewis (2013)). In the 1990s it became clear that the low number of newly discovered antibiotics could no longer overcome the increasing incidence of multi-drug resistant strains, rendering some infections untreatable by clinically approved antibiotics. Consequently, the pharmaceutical industry invested in new platforms for drug discovery, mainly based on the screening of synthetic compounds against targets identified by genomics. These screening efforts did not yield any drug with a reasonable spectrum of activity. One of the major issues was, despite the detection of *in vitro* activity against the target, the difficult penetration of the bacterial cell envelope (Payne *et al.*, 2007). These unsuccessful and costly endeavors caused the pharmaceutical industry to leave the field of antibacterial drug discovery.

1.4.1.2 Alternative sources

In the golden era of antibiotic discovery, soil-derived organisms were screened for antibacterial agents leading to the identification of about 20 different antibiotic classes. However, after some time, known compounds were rediscovered upon which this platform was abandoned. Sequencing the genome of *Streptomyces coelicolor* revealed that this organism has a far bigger potential of producing bioactive substances than the number of secondary metabolites known to be produced under standard laboratory conditions (Bentley *et al.*, 2002; Challis, 2014). An intriguing new strategy consists of looking into these silent operons. One of the first companies to explore this option was Ecopia Biosciences which, by varying the growth conditions, discovered several new secondary metabolites including a novel antibacterial agent (Banskota *et al.*, 2006). Another approach consists of cloning the silent operons into a host strain for production (Baltz, 2010) or altering expression in the original producing strain (Komatsu *et al.*, 2010; Gomez-Escribano & Bibb, 2011). Large-scale induction of such operons could provide a new platform for antibiotic discovery.

Natural products from marine sources seem to be chemically more diverse compared to terrestrial products, making oceans a good but yet underexplored niche for new antimicrobial agents (Kong *et al.*, 2010). Exploration of this niche has revealed the presence of numerous secondary metabolites, mainly produced by bacteria. Recently, several secondary metabolites produced by a coralline algal-associated *Pseudoalteromonas* strain (Tebben *et al.*, 2014) and two new antibacterials produced by a marine *Streptomyces* strain (Mondol & Shin, 2014) showing activity against *P. aeruginosa* were described, pointing to the promising potential of oceans and seas as antibacterial drug source. In addition, unculturable bacteria from different niches, unable to grow under standard laboratory conditions, proved to be very promising sources of novel secondary metabolites. For example, by exploring soil DNA, several genomic regions encoding antibacterial compounds were discovered (Gillespie *et al.*, 2002). NovoBiotic Pharmaceuticals has developed a technology to grow unculturable organisms in the lab (Kaeberlein *et al.*, 2002) which it currently uses for antibiotic drug discovery. Using this technique, novel candidate antibiotics against *S. aureus* were discovered (Peoples *et al.*, 2008).

In the 1990s, the pharmaceutical industry switched to synthetic chemical libraries as a potential source of new antibacterial drugs. However, screening of these libraries did not yield a substantial number of promising lead compounds (Payne *et al.*, 2007). To improve drug discovery, more diverse libraries should become available. Another strategy proven to be successful is the use of libraries that were initially meant for other screening purposes. For instance, screening of a library of ATP-mimicking molecules resulted in the identification of a new class of antibacterial agents (Miller *et al.*, 2009).

Another alternative source of antibacterial compounds is provided by so-called ‘repurposing’ compound libraries. These collections contain for instance compounds that have no major safety issues but failed in clinical trials, were no longer commercially favorable, or have patents that are about to expire. These libraries offer the advantage that toxicity, pharmacological and pharmacodynamical properties have already been tested (Allarakhia, 2013).

1.4.1.3 Improved screening platforms

In addition to find new sources for new antibiotics, it is important to develop reliable screening platforms which increase the chance of drug discovery. A very important parameter for discovery of drugs targeting Gram-negatives, and in particular *P. aeruginosa*, is the ability to penetrate the Gram-negative outer membrane (Lewis, 2013). A possibility is to determine a set of penetration parameters based on common properties of molecules that seem to penetrate the outer membrane effectively. Such an empirical approach worked really well in the past to define a set of parameters important for orally bioavailable compounds (Lipinski *et al.*, 2001). Based on these results, existing compound libraries can be re-evaluated. Some physiochemical properties that negatively affect outer membrane permeation are already known. For instance, comparison of 147 antibacterial active compounds revealed that drugs active against Gram-negatives usually are < 600 Da, hydrophilic (reflected by a low partition coefficient) and polar (O'Shea & Moser, 2008). In addition, presence of uncommon metal atoms improves penetration of the outer membrane. An example of such an antibiotic is AN3365, a boron-containing leucyl-tRNA-synthetase inhibitor, discussed in Section 1.4.1.3 (Sutcliffe, 2011).

To prevent rediscovery of existing classes of antibiotics, a so-called 'counterscreening' can be carried out in which the compounds are analyzed by mass and nuclear magnetic resonance spectrometry to filter out existing molecules (Silver, 2011; Lewis, 2013).

1.4.1.4 Pipeline overview

A range of new antibacterials with activity against Gram-negatives has entered clinical trials, but most of them show only modest *in vitro* activity against *P. aeruginosa* (Bassetti *et al.*, 2013; Pucci & Bush, 2013). These new antibacterial agents mostly belong to familiar classes of antibiotics such as quinolones, aminoglycosides, tetracyclines, ß-lactams in combination with ß-lactamase inhibitors and polymyxins. In addition, two compounds with a new mode of action have been developed, both acting on the membrane. Agents with reported *in vitro* anti-pseudomonal activity are listed in Table 1.1 along with their current status and identifier or reference of the indicated clinical trial. The available chemical structures are shown in Figure 1.1.

Several new **quinolones** were developed with the majority of them showing improved *in vitro* activity against Gram-positives but rather modest activity against *P. aeruginosa* (Pucci & Bush, 2013). Nemonoxacin (Adam *et al.*, 2009) and zabofloxacin (Park *et al.*, 2006) display comparable activity to that of moxifloxacin, with a minimal inhibitory concentration which inhibitied 90 % of the tested isolates (MIC_{90}) of 32 and 64 µg mL^{-1} respectively. Delafloxacin showed a comparable result to ciprofloxacin with an MIC_{90} of 0.25 µg mL^{-1} (Nilius *et al.*, 2003). Finafloxacin, a pH- activated fluoroquinolone, displayed a similar activity compared to ciprofloxacin in an acidic environment (pH 5.8 – 6.2), with an MIC_{90} of 2 µg mL^{-1} (Stubbings *et al.*, 2011). JNJ-Q2 showed comparable results to levofloxacin, but was less potent than ciprofloxacin (Morrow *et al.*, 2010). Finally, ACH-702 showed comparable results to that of ciprofloxacin with an MIC_{90} of 8 µg mL^{-1} but was metabolized quickly. Therefore, this compound was excluded for systemic application, but was licensed for use in ophthalmic infections (Pucci & Bush, 2013) (not listed in Table 1.1).

One new **aminoglycoside**, called plazomicin, has recently entered clinical trials. This antibiotic shows modest activity against *P. aeruginosa*, with an MIC_{90}-vaue of 32 µg mL^{-1} (Landman *et al.*, 2011). In addition, also the **tetracycline** eravacycline shows modest activity (16 µg mL^{-1}) against *P. aeruginosa* (Sutcliffe, 2011).

Pleuromutilines were discovered in the early 1950s but were never further developed as a consequence of their weak *in vivo* activity. In 2007, the first pleuromutulin, retapamulin, was introduced on the market for topical use (Pucci & Bush, 2013). The novel pleuromutiline AN3365 shows anti-pseudomonal activity with 90 % of the tested isolates inhibited at a concentration of 4 µg mL^{-1} (Sutcliffe, 2011). However, phase II clinical trials were terminated after development of resistant bacteria during treatment with this compound.

Several new **ß-lactams** are currently under clinical development. Among these is the monobactam named BAL30072 which is included in a clinical phase I study. This antibiotic is active against multiresistant strains, with a MIC_{90} value of 8 µg mL^{-1} (Page *et al.*, 2010). Moreover, this antibiotic is resistant to hydrolysis by metallo-ß-lactamases (MBLs). In addition, two new carbapenems, named biapenem and tomopenem, are under clinical development. Biapenem shows good *in vitro* activity against *P. aeruginosa* with 90 % of the tested isolates inhibited at a concentration of 1 µg mL^{-1} (Cheng *et al.*, 1999). Currently, a combination of biapenem with the novel ß-lactamase inhibitor RPX7009 is involved in clinical trials. No *in vitro* data on activity against *P. aeruginosa* are available for this

combination. Tomopenem showed better activity than imipenem, ceftazdime and meropenem with an MIC_{90} value of 8 µg mL^{-1} against *P. aeruginosa* strains (Koga *et al.*, 2005). Ceftolozane or CXA-101, a newly developed cephalosporin, shows good activity against *P. aeruginosa* with a MIC_{90} of 1 µg mL^{-1}, performing better than ceftazidime, imipenem and ciprofloxacin. Known resistance mechanisms against cephalosporines did not decrease activity of CXA-101 against *P. aeruginosa* (Takeda *et al.*, 2007). Since this antibiotic is susceptible to additionally acquired ß-lactamases (Livermore *et al.*, 2009), it is combined with the ß-lactamase inhibitor tazobactam. This combination, named CXA-201, completed several phase III clinical trials for intra-abdominal infections and urinary tract infections[5] and is expected to enter a phase III clinical trial against ventilator-associated pneumonia (VAP) (NCT02070757).

Several new **ß-lactamase inhibitors** are under clinical development, including avibactam (NXL104), MK-7655 and ME1071. Avibactam differs from the previous clinically approved inhibitors since it lacks a ß-lactam core. In addition, avibactam is a reversible inhibitor, representing a novel mode of action among ß-lactamase inhibitors (Ehmann *et al.*, 2012). This inhibitor displays activity against classes A and C ß-lactamases. Presently, combinations of avibactam with ceftazidime and aztreonam are enrolled in clinical trials. The combination ceftazidime-avibactam showed better activity *in vitro* compared to ceftazidime monotreatment against susceptible and multi-drug resistant *P. aeruginosa* clinical isolates (Mushtaq *et al.*, 2010; Walkty *et al.*, 2011; Levasseur *et al.*, 2012). Currently, this combination is used in several phase III clinical trials including one for treatment of hospital-acquired pneumonia (HAP) (NCT01808092). The combination aztreonam-avibactam showed modest activity against *P. aeruginosa* (Levasseur *et al.*, 2012), while the combination ceftaroline-avibactam showed very limited activity against *P. aeruginosa* with a MIC value > 32 µg mL^{-1} (Castanheira *et al.*, 2012). Therefore, these combinations will not be discussed. Another ß-lactamase inhibitor named MK-7655, similar to avibactam, was developed with activity against class A and C carbapenemases. The combination of imipenem and MK-7655 acted synergistically on three different *P. aeruginosa* isolates each overexpressing AmpC and lacking OprD (Hirsch *et al.*, 2012). Lastly, ME1071 was developed, which specifically inhibits MBLs (Ishii *et al.*, 2010). This inhibitor increased the number of susceptible MBL-producing *P. aeruginosa* strains in combination with biapenem and ceftazidime. In addition,

[5] https://clinicaltrials.gov/ct2/results?term=CXA-201&Search=Search

the novel inhibitor RPX7009, with inhibitory activity against class A and C serine ß-lactamases is tested in combination with biapenem. However, as mentioned above, no data on anti-pseudomonal activity are available for this combination therapy.

One new polymyxin, CB-182,804, was developed showing activity against *P. aeruginosa* with an MIC_{90} value of 8 µg mL^{-1} (Quale *et al.*, 2012). Synergy with rifampicin was observed, leading to a restoration of activity to polymyxin-resistant strains. In addition, two new promising membrane-acting agents both targeting outer membrane biogenesis are currently being tested in clinical trials. The first one is POL7080, a protein epitope mimetic which targets LptD, a protein involved in LPS transport (Srinivas *et al.*, 2010). This antibiotic is highly potent, with MIC values in the nanomolar range. POL7080 was licensed by Roche from the pharmaceutical company Polyphor in 2013[6]. Phase II clinical trials are currently ongoing. Secondly, ACHN-975 represents a new class of membrane-acting agents by targeting LpxC, with an MIC value below 1 µg mL^{-1} [7].

Table 1.1: Overview of anti-pseudomonal agents currently involved in clinical trials. Based on the recent review articles from Pucci & Bush (2013) and Basseti *et al.* (2013) Only compounds with reported activity against *P. aeruginosa* were retained. *In vitro* MIC values were looked up in the respective research article(s), the current status of the investigational drug was checked on https://clinicaltrials.gov.

Antibiotic	**MIC_{90} (µg mL^{-1})**	**Phase of development***	**Clinical trial identifier or reference**
Quinolones			
Nemonoxacin	32	Phase II ended, CAP	NCT01537250
Delafloxacin	0.25	Phase III recruiting, skin infections	NCT01984684
Finafloxacin	2	Phase II recruiting, UTI	NCT0192843
Zabofloxacin	64	Phase III ongoing, COPD	NCT01658020
JNJ-Q2	2	Phase II ended, skin infections	NCT01128530
		Phase II terminated, CAP	NCT01198626

[6] http://www.roche.com/media/media_releases/med-cor-2013-11-04.htm

[7] http://www.achaogen.com/antipseudomonal-programs/

Table 1.1 - continued

Antibiotic	MIC_{90} ($\mu g\ mL^{-1}$)	Phase of development*	Clinical trial identifier or reference
Aminoglycosides			
Plazomicin (ACHN-490)	32	Phase III recruiting, bloodstream infections and nosocomial pneumonia	NCT01970371
Tetracyclines			
Eravacycline (TP-434)	16	Phase III recruiting, UTI	NCT01978938
		Phase III ongoing, cIAI	NCT01844856
CB-182,804	8	Phase I	(Bassetti *et al.*, 2013)
Polymyxins			
CB-182,804	8	Phase I	(Bassetti *et al.*, 2013)
Pleuromutilins			
AN3365**	4	Phase II terminated, community acquired infection	NCT01702350
			NCT01495065
		Phase II terminated, cIAI	NCT01381562
		Phase II terminated, UTI	NCT01381549
ß-lactams			
BAL30072	8	Phase I	Bassetti *et al.* (2013)
Biapenem (RPX2003)	1	Phase I completed	NCT01702649
Tomopenem (CS-023)	8	Phase II	Bassetti *et al.* (2013)
Combination of ß-lactams and ß-lactamase inhibitors			
Ceftazidime-avibactam	< 8	Phase III recruiting, HAP	NCT01808092
Imipenem–MK-7655	≤ 4	Phase II recruiting, UTI	NCT01505634
		Phase II recruiting, cIAI	NCT01506271
Ceftolozane-tazobactam (CXA-201)	1	Phase III not yet recruiting, VAP	NCT02070757

Table 1.1 - continued

Antibiotic	MIC_{90} ($\mu g\ mL^{-1}$)	Phase of development*	Clinical trial identifier or reference
Biapenem –RPX7009	NA*	Phase I completed	NCT01772836
ME1071	/	Phase I	Bassetti *et al.* (2013)
Membrane-acting			
POL7080	0.25	Phase II recruiting, VAP and bronchiectasis	NCT02096328 NCT02096315
ACHN-975	< 1	Phase I completed	NCT01597947
		Phase I terminated	NCT01870245

*CAP, community-acquired pneumonia; UTI, urinary tract infections; COPD, chronic obstructive pulmonary disease; HAP, hospital acquired pneumonia; cIAI: complicated intra-abdominal infections; VAP: ventilator-associated pneumonia; NA, not available

** Phase II trials terminated because of development of resistant bacteria during treatment for UTI

1.4.2 Immunization and immunotherapy

An alternative strategy for treatment or prevention of *P. aeruginosa* infections is immunization of the host. This can be done passively, by using antibodies targeting specific features of *P. aeruginosa*, or actively, by administration of the antigen itself. In addition, stimulation of the innate immune system is another way to improve bacterial clearance. Below, some examples of each strategy are given.

1.4.2.1 Passive immunization

In patients already infected or colonized with *P. aeruginosa*, passive immunization or immune therapy using antibodies targeting *P. aeruginosa* can be used in combination with antibiotics. These antibodies stimulate the immune system response, improving outcome of infected patients. Several studies describe the effect of antibodies targeting specific cell-associated virulence factors such as flagella, pili and LPS, which are important in the initial attachment stage of the bacterium to the epithelial cells of the host tissue (Kipnis *et al.*, 2006).

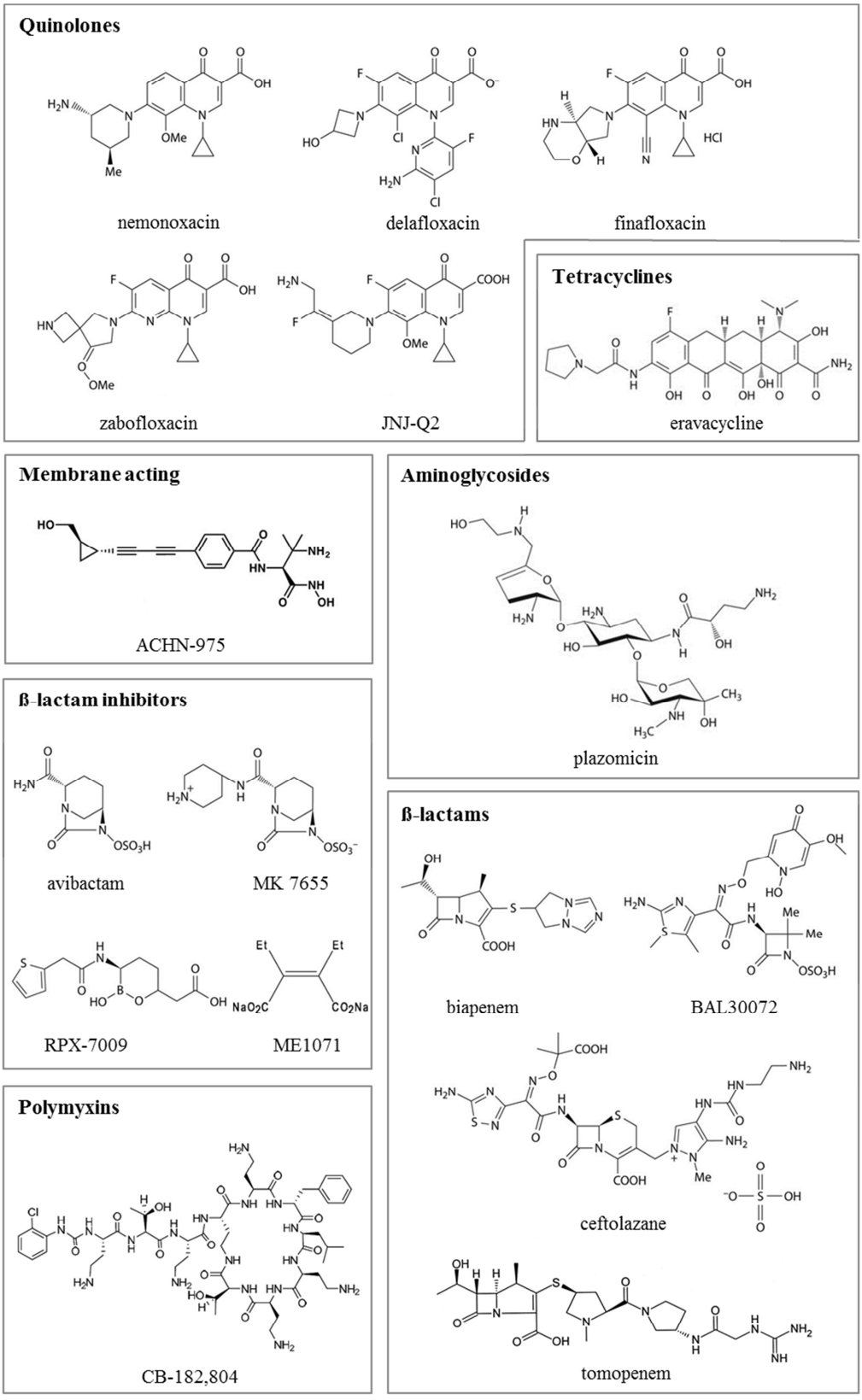


Figure 1.1: Chemical structures of new anti-pseudomonal agents as listed in Table 1.1. The structure of POL7080 has not been made available.

For instance, administration of antibodies against the **LPS** polysaccharide moiety serotype O11, which is one of the most reported serotypes among epidemiological studies, reduced the bacterial load and inflammation response upon acute pulmonary infection in immune competent (Secher *et al.*, 2011) and neutropenic mice (Secher *et al.*, 2013). Furthermore, a phase II clinical trial on critical patients suffering from nosocomial pneumonia showed an improved clinical outcome upon adjunctive treatment with the anti-LPS antibody panobacumab (Que *et al.*, 2014). Another example is provided by the anti-*Pseudomonas* antibody IgY, showing high affinity for **flagellin** (Nilsson *et al.*, 2007). This antibody prevents adherence of *P. aeruginosa* to epithelial cells, inhibiting colonization of epithelial cells. Based on phase I feasibility studies (Kollberg *et al.*, 2003), treatment of CF patients intermittently colonized by *P. aeruginosa* with IgY is permitted in Sweden (Nilsson *et al.*, 2008). Currently, a phase III trial is ongoing which aims to delay the onset of *P. aeruginosa* reinfections in CF patients which had been successfully treated for an infection (NCT01455675).

After the initial phase of infection, *P. aeruginosa* secretes several virulence factors that cause severe damage to the host tissue and play a role in invasion and further dissemination within the host (Kipnis *et al.*, 2006). Examples include proteases such as alkaline protease, hemolysines including exotoxin A and the type III secreted exotoxins ExoS, ExoT, ExoU and ExoY. Antibodies targeting these secreted virulence factors have been developed. An example includes the genetically engineered antibody against PcrV, a component of the **type III secretion system** of *P. aeruginosa*, which reduced virulence in a mouse pneumonia model (Baer *et al.*, 2009). A phase II dose-finding study on VAP patients colonized by *P. aeruginosa* showed a reduction in pneumonia incidence upon treatment with anti-PcrV (Francois *et al.*, 2012). Recently, this anti-PcrV antibody was evaluated in CF patients, and was shown to reduce inflammation compared to the control group (Milla *et al.*, 2014).

1.4.2.2 Active immunization

In theory, infections can be delayed or even prevented by immunization of the host against *P. aeruginosa* by a process called active immunization. Active immunization is carried out by exposing the host to a *P. aeruginosa* antigen to stimulate the host immune system. This can be done by administration of the purified antigen itself or by the use of DNA vaccines encoding

the *P. aeruginosa* antigen (Doring & Pier, 2008). Several antigens have proved to be successful in a number of studies.

Immunization of mice with semi-purified **exotoxin A** (Manafi *et al.*, 2009) or the combination of pure exotoxin A with an adjuvant (Pavlovskis *et al.*, 1981) significantly protected mice from induced *P. aeruginosa* burn wound infections. Another study showed that immunization of mice with the combination of purified exotoxin A and alkaline protease protected mice against gut-derived *P. aeruginosa* sepsis (Matsumoto *et al.*, 1998). Another virulence factor successful in immunization of hosts against *P. aeruginosa* infections is LPS. For instance, immunization with **LPS** protects mice from burn wound infections (Cryz *et al.*, 1984), guinea pigs from pneumonia (Pennington, 1979) and was reported to be successful in treatment of humans with burn infections (Roe & Jones, 1983). Furthermore, this treatment elicits a good immunogenic reaction in non-*Pseudomonas* colonized CF patients (Schaad *et al.*, 1991). Immunization with **outer membrane proteins** protected immunosuppressed mice from intraperitoneal challenge with *P. aeruginosa* (von Specht *et al.*, 1994; von Specht *et al.*, 1995), burn wound infections (Matthews-Greer & Gilleland, 1987), systemic infections (Gilleland *et al.*, 1984) and lung infections (Hughes & Gilleland, 1995) while immunized rats were protected against pulmonary infections (Gilleland *et al.*, 1988). Active immunization with **flagellin** was also reported in several studies. For example, administration of recombinant type A flagellin protected mice with burn injuries against lethal *P. aeruginosa* infections (Faezi *et al.*, 2014). In addition, several vaccines have been described in which two or more bacterial antigens are conjugated. Examples include a fusion protein of OprF epitope 8, OprI, and type A and B flagellins (Weimer *et al.*, 2009) and a conjugate of alginate and flagellin (Campodonico *et al.*, 2011). Active immunization can also be achieved by using whole, attenuated *P. aeruginos*a strains. It was shown that immunization with a live-attenuated *Pseudomonas* vaccine protected neutropenic mice against lethal pneumonia infections induced by both homologous and heterologous strains (Kamei *et al.*, 2013).

Several clinical trials have been conducted to assess the efficacy of vaccination in CF patients. Vaccines were tested for their capability to prevent or reduce established *P. aeruginosa* infections in the CF airway. Three of them are reviewed in Johansen & Gotzsche (2013) and include two large trials, one with a vaccine composed of flagellin proteins (Doring *et al.*, 2007). The other one tested the efficacy of a vaccine known as Aerugen of which the composition was not disclosed (unpublished data cited by Johansen & Gotzsche (2013)). A

third small trial was conducted with a polysaccharide vaccine containing 16 different serotypes (Langford & Hiller, 1984). Analysis of these data did not show effective prevention of *P. aeruginosa* infection (Johansen & Gotzsche, 2013).

1.4.2.3 Supplementation of the innate immune system

Another possibility to improve bacterial clearance by the host is stimulation of the innate immune response. For instance, one of the responses of the airway to bacterial infection, defective in CF patients, is the production of hypothiocyanite ($OSCN^-$), which acts bactericidal (Moskwa *et al.*, 2007). In addition, it has been shown that lactoferrin, a constituent of human external secretions, prevents biofilm formation of *P. aeruginosa* (Singh *et al.*, 2002). Treatment of CF patients with nebulized hypothiocyanite in combination with lactoferrin showed good results and was recently approved as an orphan drug known as Meveol (Hurley *et al.*, 2012).

1.4.3 Targeting non-essential processes

Several processes that are not essential for bacterial viability contribute to the severe outcome of infections caused by *P. aeruginosa.* One of them is expression of a wide arsenal of virulence factors, of which many are under the control of QS. Secondly, the formation of biofilms by this pathogen seriously hampers accurate eradication (described in Section 1.3.2). Treatments aimed at reducing virulence or preventing biofilm formation would therefore greatly help in improving patients' outcome upon infection by *P. aeruginosa*. In theory, since these processes are not essential for viability of the bacterium *in vitro*, targeting them may impose a weaker selection pressure *in vivo*, decreasing the rate of resistance development. These strategies attenuate the pathogen, giving the immune system the opportunity to eliminate the bacterial population from the human body. Some examples are described below.

1.4.3.1 Targeting virulence

Many anti-virulence strategies targeting *P. aeruginosa* have been described (for an overview see Hurley *et al.* (2012)) and some examples will be discussed briefly. Virulence of *P. aeruginosa* can be altered by targeting specific cell-associated virulence factors. For instance,

it was shown that flagellum-deficient mutants are defective in causing acute infections (Feldman *et al.*, 1998), and mutants lacking pili caused less severe infections in mice (Tang *et al.*, 1995). Furthermore, the functional expression of the type III secretion system is associated with increased mortality in hospital patients (Roy-Burman *et al.*, 2001). These cell-associated virulence factors can be targeted by immunization as discussed in Section 1.4.2.

Another strategy to reduce virulence consists of reducing the expression of virulence factors. The expression of some of these factors is regulated by QS, a bacterial communication system based on population density through the production and detection of signaling molecules. The concentration of signaling molecules rises upon increasing population size and, once a certain threshold is reached, expression of specific genes, including some involved in virulence, is coordinated within the bacterial community (Waters & Bassler, 2005). *P. aeruginosa* contains at least three different QS systems of which the Las and Rhl pathways use N-acylhomoserine lactones (AHLs) as signaling molecules (Winson *et al.*, 1995), the alkylquinoline pathway uses 2-heptyl-3-hydroxy-4-quinolone (Diggle *et al.*, 2006). The latter is also known as the pseudomonas quinolone signal. Several strategies have been described to interfere with QS, often referred to as 'quorum quenching' (QQ).

One way to achieve QQ is by preventing the accumulation of signal molecules within the bacterial cell through interference with signal production. An example is given by the small molecule triclosan, which binds to FabI, an enoyl-acyl carrier protein reductase, with an essential role in the production pathway of AHLs (Hoang & Schweizer, 1999). Secondly, degradation of the signaling molecules itself represents an effective way to prevent signal molecule accumulation. For this purpose, enzymes that degrade the autoinducer AHL can be used, such as AHL lactonase or acylase (Czajkowski & Jafra, 2009). For example, a *P. aeruginosa* strain overproducing an AHL acylase was shown to be less virulent in the fast-killing assay on *Caenorhabditis elegans*, while a *P. aeruginosa* mutant lacking this enzyme showed higher virulence in a slow-killing assay. In addition, exogenous addition of the purified acylase reduced pathogenicity of *P. aeruginosa* (Papaioannou *et al.*, 2009). More recently, it was shown that overexpression of the AHL lactonase AiiM reduces cytotoxicity of *P. aeruginosa* in a mice model of acute pneumonia (Migiyama *et al.*, 2013). Another approach lies in antagonizing the mode of action of the produced signaling molecules. This can be achieved by administration of synthetic AHL analogues (Smith *et al.*, 2003) that interfere with binding of the signaling molecules to their receptor or that reduce the

concentration of available receptors within the bacterial cell. An example of QS inhibition by an AHL mimic is given by administration of furanone C-30, a synthetic derivate of natural furanone compounds, which reduced QS of *P. aeruginosa* in a mouse pulmonary infection model hereby allowing the immune system to clear the infection (Hentzer *et al.*, 2003). Also non-natural AHL-mimics have been synthetized. An example is given by a structurally novel class of chimerics composed of the natural tail region of the native signaling molecule of the LasR-system and a non-natural homoserine lactone head group. Administration of these AHL-mimics reduced the production of virulence factors of *P. aeruginosa in vitro* (Hodgkinson *et al.*, 2012).

Lastly, virulence of *P. aeruginosa* can be decreased by interfering with iron uptake and metabolism. This metal is essential for growth but is present in low amounts at the site of infection. Therefore, *P. aeruginosa* produces iron chelators with pyoverdine the most important one under iron limiting conditions (Cornelis & Dingemans, 2013). Repressing the production of pyoverdine, by targeting the sigma factor PvdS which among others controls genes necessary for biosynthesis of this iron chelator, was shown to reduce virulence of *P. aeruginosa* in a mouse model (Imperi *et al.*, 2013). Alternatively, the redox inactive iron mimetic gallium can be used to interfere with iron uptake and metabolism (as reviewed by Minandri *et al.* (2014)). Combining gallium with the pyochelin siderophore further improved the anti-pseudomonal activity of gallium by increasing its intracellular concentration (Frangipani *et al.*, 2014).

1.4.3.2 Targeting biofilms

QS plays an important role in the formation and dispersion of biofilms (Harmsen *et al.*, 2010). Consequently, by interfering with this process, formation of biofilms is prevented. Strategies to disrupt QS were discussed in the previous Section. Some additional approaches to prevent or eradicate biofilms without targeting essential processes are discussed below.

A first strategy entails prevention of biofilm formation. An example of this strategy consists of blocking the sugar-specific binding sites of lectins. Lectins are outer membrane proteins with specific sugar-binding sites that allow bacteria to cross-link and form biofilms. By adding specific competitive inhibitors of the lectins LecA and LecB, biofilm formation of *P. aeruginosa* was prevented (Johansson *et al.*, 2008; Kadam *et al.*, 2011). Another way to

prevent biofilm formation is by interfering with iron uptake, which is important in the formation of these multicellular structures (Banin *et al.*, 2005). For example, addition of iron chelators prevented biofilm formation in aerobic as well as anaerobic environments (Singh *et al.*, 2002; O'May *et al.*, 2009). The metal gallium, which is very similar to iron, interfered with biofilm formation by hindering the uptake of iron (Kaneko *et al.*, 2007). Prevention of *P. aeruginosa* biofilm formation is also achieved by addition of D-amino acids, which probably replace D-alanine in the peptide side chain of peptidoglycan. How exactly this influences biofilm formation in *P. aeruginosa* was not reported (Kolodkin-Gal *et al.*, 2010).

Secondly, when biofilms are already established, they can be disrupted by adding agents that promote dispersion. For instance, the small fatty acid messenger cis-2-decenoic acid induces dispersion of established biofilms formed by many bacterial species, including *P. aeruginosa* (Davies & Marques, 2009). The addition of the NO donor sodium nitroprusside (Barraud *et al.*, 2006), and high-affinity ligands of LecB (Johansson *et al.*, 2008) also efficiently disperse *P. aeruginosa* biofilms. Ginseng extract also prevented the formation of biofilms. Treatment of biofilms with alginate lyase loosens the biofilm matrix, making biofilm cells more susceptible to antibiotic killing (Alkawash *et al.*, 2006).

So far, only a limited number of *in vivo* and clinical studies have demonstrated improved treatment of biofilm infections, as listed in Romling & Balsalobre (2012).

1.4.4 Bacteriophages

A promising therapeutic option is the use of bacteriophages, bacteria-specific viruses capable of killing their particular host pathogen. One of the first publications on bacteriophages originates from 1917, were D'Herelle describes the isolation of 'an invisible microbe endowed with antagonistic properties towards a pathogenic bacillus' from stools of people recovering from bacterial dysentery (D'Herelle, 2007). A few years later, the first report on the use of phages as an antibacterial therapy was published (Bruynoghe & Maisin, 1921) and many firms produced phage preparations to treat bacterial infections (Hausler, 2006). However, after world war II, research and clinical use of phage therapy was abandoned because of the introduction of antibiotics in combination with the mixed successes obtained with phages. Only in countries of the former Soviet Union, development of phage therapy continued, and was used as a conventional treatment of bacterial infections (Kutateladze &

Adamia, 2010; Viertel *et al.*, 2014). Publications on successful use of phages in the 1980s (Smith & Huggins, 1982; Smith & Huggins, 1983; Smith *et al.*, 1987) along with the development of antibiotic resistance in the early 1990s, rekindled the interest in phage therapy.

Phage therapy possesses several advantages but also disadvantages over antibiotic therapy as discussed in Hausler (2006) and Lu & Koeris (2011). Phages are highly specific to the target organism without causing a negative effect on the commensal microbiota hereby preventing side effects often associated with conventional antibiotic therapies. As a consequence, no selection of resistances in these commensal bacteria occurs. Nevertheless, this great specificity of phages becomes a disadvantage when the identity of the infecting bacterial species is unknown or when the infection is polymicrobial. Another advantage is provided by the fact that phages are active against bacterial strains resistant to multiple antibiotic classes. However, bacteria also develop resistance against phages through several different mechanisms (as reviewed by Labrie *et al.* (2010)). Other disadvantages associated with phage therapy include challenges associated with phage delivery. Phages are removed by the immune system before they reach the site of infection, especially when administered systemically. In addition, antibodies against these phages are produced, limiting the number of systemic treatments that can be carried out with one specific phage formulation. Therefore, many studies focus on treatment of non-systemic infections such as wound or ear infections. In addition, bacteria residing inside human cells are inaccessible to phages, making them unsusceptible to phage therapy. Endotoxins and antigens, which may be present in phage formulations as a result from the manufacturing process, or which are released upon phage-mediated lysis of bacterial cells stimulate an inflammatory response leading to significant morbidity. Interestingly, phages can be engineered to overcome some of the problems related to phage therapy (Lu & Koeris, 2011). In addition, by adding monomethoxypolyethylene glycol to the phage surface, the immune response of the host is delayed (Kim *et al.*, 2008). Another example is the use of lysis-deficient engineered phages in order to prevent release of toxic substances due to bacterial lysis (Paul *et al.*, 2011).

Several phage therapies based on the use of whole phages were evaluated in clinical trials. For example, a phase I/II clinical trial demonstrated the efficacy and safety of a phage cocktail targeting resistant *P. aeruginosa* strains in chronic otitis (Wright *et al.*, 2009). Another clinical trial proved the safe application of phages for treatment of venous leg ulcers (Rhoads

et al., 2009). Other trials are ongoing, including evaluation of phage therapy on *P. aeruginosa* burn wound infections (NCT02116010) and non-healing wounds (NCT00945087). Currently, no phage therapy is approved for clinical use in humans, although some applications were approved for use in the food (ListShield™ and LISTEX™ P100) and agricultural (Omnilytics' AgriPhage™) industry (Lu & Koeris, 2011; Viertel *et al.*, 2014).

Phages can also be engineered to deliver genes or lethal substances to the site of infection. For instance, treatment of *P. aeruginosa* infected mice with a phage encoding a restriction endonuclease proved to be successful (Hagens *et al.*, 2004). Alternatively, phage-derived lytic enzymes can be used as antibiotic substances themselves. An example is provided by the endolysins, target-specific enzymes that degrade the bacterial cell wall. However, in case of *P. aeruginosa*, a Gram-negative bacterium, the action of endolysins is hindered by the presence of the outer membrane (Walmagh *et al.*, 2013). This problem can be overcome by combining an endolysin with a targeting peptide, which transports the endolysin through the outer membrane of Gram-negative bacteria (Briers *et al.*, 2014c).

1.5 Concluding remarks

P. aeruginosa is a versatile human pathogen, especially known to infect persons in which the local and/or systemic immune system is impaired or does not function properly. As the number of people receiving immunosuppression and invasive treatments increases, nosocomial *Pseudomonas* infections become more prevalent. *P. aeruginosa* has the remarkable ability to acquire additional resistance mechanisms, and consequently, infections are increasingly caused by strains resistant to one or more antibiotic classes. In critical cases, infecting strains are resistant to all currently available antibiotics. In general, the need for new antibacterial agents is further stressed by the World Health Organization, which identified multi-drug resistant bacteria as a serious threat to human health[8]. Importantly, without antibacterial agents, some medical interventions become impossible as they rely on the protection offered by antibiotics.

Currently, some new anti-pseudomonal agents are under clinical investigation but they mainly belong to existing antibiotic classes to which resistance develops within short time frames

[8]WHO (2014) Antimicrobial resistance: global report on surveillance

after introduction of the new antibiotic on the market. Therefore, new discovery strategies need to be followed to increase chances of discovering mechanistically novel antibacterial agents. Exploration of alternative natural sources combined with improved screening platforms, which filter out already discovered structures, might lead to identification of new promising antibacterial substances. In addition, new treatment strategies, which slow down the rate of resistance development, and development of alternative approaches such as phage and immunotherapy, are necessary to win the battle against infectious diseases.

Even in the absence of antibiotic resistance, infections caused by *P. aeruginosa* may be difficult to treat. This can be attributed to the formation of biofilms and the presence of persister cells, which prevent accurate treatment of bacterial infections. Combining an antibiotic with a compound targeting a non-essential process such as biofilm or persister formation may increase efficacy of treatment, hereby avoiding relapse of infection.

Taken together, there is an urgent need for new anti-pseudomonal agents and treatment strategies. Special efforts should be made to improve eradication of infecting bacterial populations in order to prevent recurring infections. This eliminates the necessity to expose the organism to another round of antibiotics and, as a consequence, decreases chances of resistance development against the used antibacterial agent.

Chapter 2

Persister cells, clinical importance and treatment strategies

2.1 Introduction

Exactly 70 years ago, it was noticed that upon treatment of antibiotic-sensitive *Staphylococcus aureus* cultures with penicillin, a small fraction of cells survived. When these cells were re-cultured, again, a small fraction of surviving cells was observed, excluding the involvement of resistance. Therefore, these cells were called persisters (Bigger, 1944). At the time, the clinical relevance was overlooked, which resulted in little attention from the scientific community for persister cells. In the beginning of the 21st century it was reported that persister cells are also present in biofilms, which are held responsible for the recalcitrant nature of infections (Brooun *et al.*, 2000; Spoering & Lewis, 2001), leading to renewed interest in these antibiotic-tolerant cells.

The presence of persister cells in an isogenic bacterial population typically results in a biphasic killing pattern upon treatment with a bactericidal antibiotic, with non-persisters exhibiting fast killing kinetics and persisters dying slowly. During treatment, persister cells are not able to grow, but upon a drop in antibiotic concentration, persisters revert to regular cells and regrow into a susceptible population (Cohen *et al.*, 2013)(Figure 2.1). Formation of persister cells is dependent on the growth phase. Typically, persister levels are low in early exponential phase, increase during late-exponential phase and reach a maximum during stationary phase (Keren *et al.*, 2004a). Initially, the persister population was considered homogeneous, but it is becoming increasingly clear that different subpopulations of persisters can be identified. It was suggested that the diversity in persisters results from the fluctuations and variability in cellular processes. This means that persisters can arise as a consequence of different mechanisms, giving rise to distinct persister populations, each with their particular

tolerance mechanisms (Allison *et al.*, 2011b). This hypothesis is further supported by the observation that different levels of persisters are detected upon treatment of a population with antibiotic belonging to different classes (Keren *et al.*, 2004a; Goneau *et al.*, 2014).

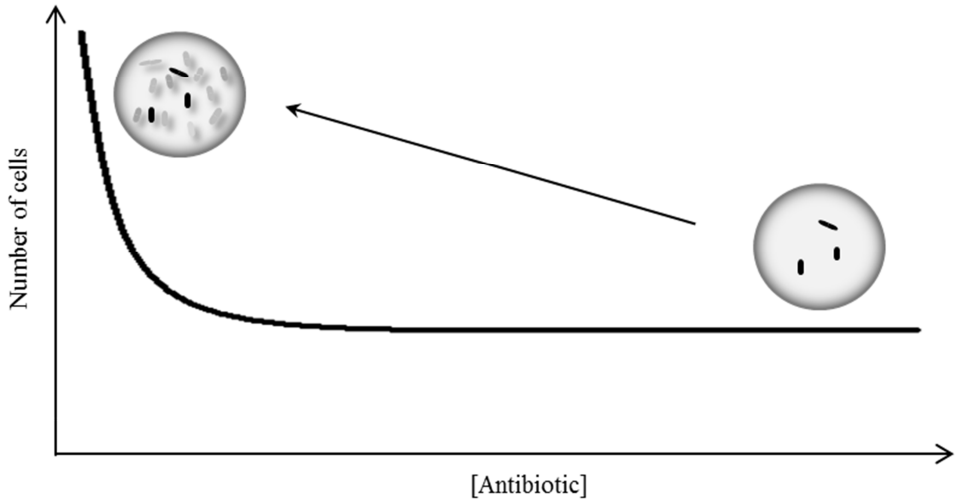


Figure 2.1: Biphasic killing pattern of a sensitive bacterial population in response to a bacteriocidal antibiotic. Addition of increasing concentrations of a bactericidal antibiotic initially leads to a sharp decrease of the number of viable cells. However, a further increase of antibiotic concentration does not lead to additional killing. A small fraction of cells, the persister cells (black), remain viable. If the antibiotic pressure drops, persister cells revert to growing cells and give rise to a susceptible population (as depicted by the arrow), which again contains a small fraction of persisters. Adapted from Kint *et al.* (2012).

2.2 Characteristics of persister cells

Traditionally, persisters are considered to be non-dividing, metabolically quiescent cells in which inactivity of the antibacterial targets impairs the bactericidal effect (Bigger, 1944). This is supported by the observation that genes involved in metabolic pathways, energy production and non-essential genes are downregulated, lending support to a dormant state of persisters (Keren *et al.*, 2004b; Shah *et al.*, 2006; Keren *et al.*, 2011; Wood *et al.*, 2013). However, it is becoming clear that the persistent state is far more complex than just a passive dormant state. A first clue was provided by the observation that an isolated dormant population, based on activity of the growth-dependent *rrnB* P1 promotor, is not entirely composed of persister cells (Shah *et al.*, 2006). Moreover, it was shown that *Escherichia coli* persister cells still display some degree of translation (Gefen *et al.*, 2008). Additional evidence supportive of this hypothesis was provided by the observation that a dormant state is not sufficient or required

for a cell to become persistent (Orman & Brynildsen, 2013). Furthermore, no correlation between the growth rate of an individual cell and persistence was observed in *Mycobacterium* (Wakamoto *et al.*, 2013).

Persister cells are already present in the population before antibiotic treatment (Balaban *et al.*, 2004) were they can serve as insurance for the entire population in case of a sudden antibiotic attack (Kussell *et al.*, 2005). Combined with the fact that persister cells comprise a very small fraction of an isogenic population (Keren *et al.*, 2004a), it was hypothesized that formation of persisters occurs through stochastic processes (Lewis, 2010). However, the formation of persisters can also be triggered by environmental cues such as signaling molecules (Moker *et al.*, 2010; Vega *et al.*, 2012), stress (Dorr *et al.*, 2009; Wu *et al.*, 2012; Kwan *et al.*, 2013; Goneau *et al.*, 2014) and entry into stationary phase (Keren *et al.*, 2004a). This points to the involvement of a deterministic compound in persister formation. Based on these observations, the following model was proposed. The mean expression level of a dedicated persister protein in a population, set by environmental conditions, represents the deterministic compound. Random stochastic fluctuations around this mean will give rise to a small fraction of cells in which the persister protein exceeds a certain treshold, causing these cells to switch to the persistent state (Kint *et al.*, 2012). This theory is supported by the observation that *E. coli* cells become persistent when the intracellular concentration of the toxin HipA reaches a certain threshold (Rotem *et al.*, 2010). More recently, it was shown that individual cell fate under antibiotic treatment conditions was linked to stochastic expression of the isoniazid-activating enzyme catalase peroxidase (KatG) in *Mycobacterium* (Wakamoto *et al.*, 2013).

2.3 Clinical importance

Persister cells comprise a small subfraction of a genetically homogeneous population that remains viable during treatment with high doses of antibiotics. It is generally assumed that, upon successful treatment with a bactericidal antibiotic, the host immune system is able to cope with this small fraction of surviving cells. However, in situations where bacteria are able to evade the immune system, persisters may become a threat to human health (Gefen & Balaban, 2009). A causal link between persister cells and the recalcitrant nature of infections has been provided, pointing to the *in vivo* relevance of these antibiotic-tolerant cells. Below, evidence in support of the *in vivo* relevance of persister cells and situations in which persister

cells become a threat for human health are discussed. In addition, the role of persisters in development of resistance and the interplay between persistence and resistance mechanisms is described.

2.3.1 *In vivo* selection for high persistence

In vivo relevance has been provided for the role of *Pseudomonas aeruginosa* persister cells in chronic infections (Mulcahy *et al.*, 2010). Comparison of early and late isolates of a chronically infected cystic fibrosis (CF) patient taken at the age of 8 and 96 months respectively, showed a 100-fold increase in persister cells. This was confirmed upon examination of isolates from additional CF patients. This result indicates that prolonged periodical administration of antibiotics selects for strains capable of producing substantial higher levels of persisters, pointing to the importance of persister cells in the recalcitrant nature of infection. Characterization of the persister fraction of uropathogenic *E. coli* strains isolated from same-strain acute and recurrent infections showed a higher survival of the latter when exposed to antibiotic treatment, pointing to selection for a higher persister level as the infection becomes more persistent (Goneau *et al.*, 2014). The presence of persister cells is not restricted to prokaryotes as they were also detected in biofilms formed by the eukaryotic pathogen *Candida albicans* (LaFleur *et al.*, 2006). Similarly, when comparing early and late isolates from patients chronically infected with *C. albicans*, a significant increase in persister level was observed for late-stage isolates (Lafleur *et al.*, 2010).

2.3.2 Clinical implications of persister cells

In specific cases, persister cells become a threat to human health (Gefen & Balaban, 2009). The first example includes persons in which the immune system is suppressed or (partially) impaired. With the many advances that were made in medical technology, the number and expected lifetime of such patients has increased. Here, the surviving persisters are not removed because of a malfunctioning host immune system, and may form a reservoir of viable bacteria able to re-establish infection. Examples include patients receiving chemotherapy, immunosuppressed transplant recipients, HIV patients, CF patients and patients in intensive care units.

Secondly, the presence of persisters becomes problematic in situations where pathogens evade the host immune system by residing at places which are poorly accessible. Examples include the central nervous system (Honda & Warren, 2009), the gastro-intestinal tract (Peterson *et al.*, 2000) and cells of the immune system. An example of the latter is provided by the Gram-negative pathogen *Salmonella enterica*. This bacterium can enter and replicate within macrophages (Haraga *et al.*, 2008), specialized white blood cells involved in the innate immune system of the host. It was shown that immediately after uptake into macrophages, a non-replicating population was formed, which could not be cleared by antibiotic treatment of infected mice. Experiments indicated that these remaining cells represent persisters, tolerant to multiple antibiotic classes (Helaine *et al.*, 2014). Similar results were obtained by Kaiser and coworkers using an intragastric mouse infection model where, after antibiotic treatment, a small fraction of slow-growing multi-drug tolerant bacteria was recovered (2014). A second example includes *Mycobacterium tuberculosis*, a facultative intracellular pathogen which can replicate within macrophages (Vergne *et al.*, 2004). According to the WHO[9], it is estimated that 1/3rd of the human population is latently infected by *M. tuberculosis*, with 10 % of them eventually developing an acute infection due to regrowth of non-replicating bacteria, resembling persister cells, present in the human body.

Lastly, persister cells can be present in specific niches within the human body that provide protection against components of the host immune system. An example is provided by persister cells residing in biofilm structures (Lewis, 2005). Biofilms are multicellular structures of bacterial cells embedded in a self-produced extracellular matrix adherent to an inert or living surface (Costerton *et al.*, 1999). Within these biofilms, persister cells are protected against the action of the host immune system by the extracellular matrix which contains among others different polysaccharides, proteins and DNA (Mann & Wozniak, 2012). It was shown that the polysaccharide alginate protects *P. aeruginosa* cells from the action of neutrophils and macrophages. These immune cells accumulate at the biofilm surface but are not able to engulf the bacterial cells. The mechanism by which alginate exerts its action is still unknown (Leid *et al.*, 2005; Mulcahy *et al.*, 2013). The extracellular DNA (eDNA) present in the matrix also plays an immunoprotective role. In *P. aeruginosa*, it was shown that eDNA is able to bind antimicrobial peptides (AMPs) and metal cations. The latter activate expression of genes including the ones responsible for modification of the lipid A

[9] http://www.who.int/mediacentre/factsheets/fs104/en/

part of lipopolysaccharide and genes responsible for spermidine synthesis. In this way, the negative bacterial surface charge is masked, protecting the outer membrane against AMPs (Lewenza, 2013). The matrix also offers protection against the humoral immune system. A study showed that penetration of antibodies is prevented by the biofilm matrix (de Beer *et al.*, 1997). Not only the matrix but also production of quorum sensing (QS)-regulated products contributes to tolerance of biofilms to the immune system. In *P. aeruginosa*, upon encountering polymorphonuclear neutrophils, synthesis of rhamnolipid is activated through QS, which eliminates phagocytes (Alhede *et al.*, 2009). In addition, expression of the virulence factors alkaline protease and elastase is upregulated during the biofilm mode of growth. These are capable of inactivating complement (Kharazmi, 1991).

2.3.3 Persistence and its role in resistance

Besides hindering effective clearance of bacterial populations, persister cells also contribute to the development of resistance. In theory, they prolong the stay of the pathogen in the human body, increasing exposure to antibiotic agents leading to a higher possibility of developing additional resistance mechanisms (Levin & Rozen, 2006). Furthermore, crosstalk between resistance and persistence has been reported in *P. aeruginosa* (De Groote *et al.*, 2011). In this study, the authors show that strains resistant to fosfomycin, either by *fosA* overexpression or by *glpT* mutation, have a decreased persister fraction upon ofloxacin treatment. On the other hand, it was shown that induction of an efflux pump, a classical resistance mechanism, increased levels of persister formation in *E. coli* (Wu *et al.*, 2012).

2.4 Mechanisms of persister formation in *Pseudomonas aeruginosa*

The molecular mechanism underlying persister formation in *P. aeruginosa* is largely unknown and only few genetic determinants have been described to influence the level of persister cells in this pathogen (as reviewed in Fauvart *et al.* (2011)). These genes are mainly involved in processes related to stress responses. How exactly they regulate persister formation is unknown but it seems that some of the genes are somehow correlated. Some of the mechanisms implicated in *P. aeruginosa* persistence were also shown to influence persistence in other bacterial species. The most important mechanisms in *P. aeruginosa*,

along with their similarities and differences in other bacterial species, are briefly discussed below.

2.4.1 The alternative sigma factors RpoS and RpoN

Bacterial cells possess several sigma factors, constitutively active or activated under particular conditions, which determine the specificity of RNA polymerases. In this way, expression of proteins, needed in specific stress conditions, is regulated (Borukhov & Nudler, 2003; Kazmierczak *et al.*, 2005)

The alternative sigma factor RpoS is activated upon entry in the stationary phase, inducing expression of genes required to cope with the low-nutrient conditions encountered in the stationary phase. In addition, this sigma factor is also involved in regulation of virulence and specific stress responses (Suh *et al.*, 1999). An *rpoS* mutant showed an increased susceptibility towards antibiotics (Murakami *et al.*, 2005) indicating that RpoS positively influences antibiotic tolerance.

The sigma factor RpoN, which regulates several processes needed for bacteria to adapt to unfavorable environmental conditions, is involved in the regulation of virulence factors and negatively regulates QS (Totten *et al.*, 1990; Cases & de Lorenzo, 2001; Heurlier *et al.*, 2003). A *rpoN* mutant showed a higher survival rate upon antibiotic treatment compared to the wild-type strain (Viducic *et al.*, 2007), pointing to a negative effect of RpoN on antibiotic tolerance.

2.4.2 Quorum sensing

The persister fraction of *E. coli*, *P. aeruginosa* and *S. aureus* species sharply increases at mid to late-exponential phase. Therefore, it was postulated that QS may play a role in the formation of persisters (Keren *et al.*, 2004a). Addition of spent growth medium to growing cells did not increase the persister fraction of *E. coli* or *P. aeruginosa*, suggesting that QS is not involved (unpublished result cited by Lewis (2007)). However, a few years later, several reports indicate the involvement of QS in *P. aeruginosa* antibiotic tolerance (Kayama *et al.*, 2009; Moker *et al.*, 2010; Que *et al.*, 2013).

P. aeruginosa possesses three different QS systems, named the Las, Rhl and alkylquinoline pathways. The first two use AHLs as signaling molecules, which are synthetized by LasI and RhlI respectively, and sensed by LasR and RhlR (Waters & Bassler, 2005). The third system uses the *Pseudomonas* quinolone signal as signaling molecule (Diggle *et al.*, 2006) The antibiotic tolerance of *lasR* and *lasI* mutants was significantly reduced compared to the parental strain, while overexpression of *rpoS* restored the persister fraction of the *lasR* mutant to wild-type level (Kayama *et al.*, 2009). Addition of the quorum-sensing-related signaling molecules pyocyanin or the AHL 3-oxododecanoyl-homoserine lactone increased the persister fraction (Moker *et al.*, 2010), further supporting the positive influence of quorum sensing on antibiotic tolerance. A first insight into the role of QS in antibiotic tolerance was provided by Que and colleagues (2013). They showed that the small volatile molecule 2-AA, of which the production is controlled by the QS regulator MfvR, increases the number of antibiotic tolerant cells by decreasing the transcription of genes involved in the translational capacity of the cell. Importantly, 2-AA also increased persister formation of *Burkholderia thailandensis* and *Acinetobacter baumannii,* pathogens frequently isolated together with *P. aeruginosa* (Harrison, 2007; Rezaei *et al.*, 2011).

So far, no reports show the involvement of QS in other bacterial species. However, intra-species QS was indicated to play a role in persistence in different bacterial species. For instance, mutant cells of the Gram-positive *Streptococcus mutans*, unable to respond to the stress-inducible pheromone named competence-stimulating peptide, produced less persisters (Leung & Levesque, 2012). Another example is 2-AA, produced by *P. aeruginosa*, which increases the persister fraction of *Burkholderia thailandensis* and *Acinetobacter baumannii* (Que *et al.*, 2013) as described above. Recently, an additional example was reported in which pyocyanin, produced by *P. aeruginosa***,** plays in role in the formation of antibiotic-tolerant cells in *Acinetobacter baumannii*. Pyocyanin induces protection against oxidative stress through elevation of the levels of detoxifying enzymes (Bhargava *et al.*, 2014).

In *E. coli*, another form of chemical signaling is likely involved in the formation of persister cells. Upon sensing nutrient limitation, *E. coli* cells produce the signaling molecule indole (Han *et al.*, 2011). It was shown that upon addition of this molecule to the growth medium, the fraction of multidrug-tolerant persisters increased. Accordingly, a mutant defective in indole synthesis showed a decreased persister fraction. The authors showed that indole activates the OxyR and phage shock pathways in a fraction of cells, leading to the formation

of a subpopulation of persisters (Vega *et al.*, 2012). Recently, contrasting results were published, showing that the level of persisters and the concentration of indole are inversely proportional. Overexpression of the toxin YafQ increased persistence through reduction of TnaA, leading to lower levels of indole (Hu *et al.*, 2014). Importantly, these results connect toxin-antitoxin (TA) modules with chemical signaling.

2.4.3 Stringent response

When nutrients are limiting, the alarmone (p)ppGpp, a mixture of guanosine 5'-triphosphate 3'-diphosphate (pppGpp) and guanosine 5'-diphosphate 3'-diphosphate (ppGpp), accumulates within the cell, inducing changes in gene expression to ensure survival of the cells. Synthesis and degradation of (p)ppGpp is regulated by the synthetase RelA and the bifunctional enzyme SpoT, acting as a synthetase or hydrolase depending on the conditions (Potrykus & Cashel, 2008). Genes involved in or linked to the stringent response have been implicated to play a role in *P. aeruginosa* persistence (Viducic *et al.*, 2006). A *spoT* mutant showed higher survival rates compared to the wild type, in contrast to a *relA* mutant, displaying reduced survival upon antibiotic treatment. The survival rate was correlated with the intracellular level of (p)ppGpp, with high concentrations leading to better survival upon antibiotic exposure (Viducic *et al.*, 2006). This is supported by the observation that a *dksA* mutant, showing increased tolerance against antibiotics, possesses high intracellular (p)ppGpp levels. DksA interacts with RNA polymerases in the presence of (p)ppGpp, hereby increasing binding to rRNA promoters, which negatively affects rRNA transcription. More recently, the role of the stringent response in *P. aeruginosa* persister formation was further elucidated (Nguyen *et al.*, 2011). The authors showed that activation of the stringent response upon starvation leads to suppression of oxidative stress by inducing antioxidant defenses and restricting the production of pro-oxidants. The role of oxidative stress in persistence is discussed below (see Section 2.4.4).

In *E. coli*, the stringent response was also shown to mediate antibiotic tolerance. Inactivation of the stringent response caused a decrease in the number of persister cells and was accompanied by reduced catalase and increased intracellular OH• levels, indicating that the stringent response also controls oxidative stress in this bacterium (Nguyen *et al.*, 2011). In addition, the stringent response has also been linked to toxin-antitoxin (TA) modules in *E. coli*. TA modules consist of a stable toxin, targeting essential processes in the bacterial cell,

and an unstable antitoxin, which counteracts the effects of its cognate toxin (Yamaguchi *et al.*, 2011). In the presented model, high levels of (p)ppGpp induce degradation of antitoxins through the action of the Lon-PolyP complex, rendering the toxins active. These activated toxins inhibit cell growth, causing insensitivity of the cells towards the bactericidal action of antibacterial agents (Maisonneuve *et al.*, 2013). Very recently, evidence was provided for a role of the stringent response through activation of TA modules in the intracellular pathogen *S. enterica*. A *relAspoT* double mutant, defective in (p)ppGpp synthesis, and a *lon* mutant, which is not able to degrade class II antitoxins, formed less persisters upon uptake into macrophages. Additionally, it was shown that 14 TA modules were upregulated in a *relAspoT*-dependent manner (Helaine *et al.*, 2014). According to the TA database (Shao *et al.*, 2011), the *P. aeruginosa* genome contains 7-12 type II TA loci, depending on the strain. A triple knock-out mutant lacking three TA modules did not show any difference in antibiotic tolerance (unpublished results cited by Mulcahy *et al.* (2013) although it is possible that more TA modules need to be removed to observe an effect on the persister fraction (Maisonneuve *et al.*, 2011). In *P. putida*, the toxin GraT slows down growth, permeabilizes the membrane and causes increased tolerance to antibiotic treatment (Tamman *et al.*, 2014). To date, no peer-reviewed journal articles were published regarding the role of TA-modules in *P. aeruginosa* persistence but it is clearly a topic that warrants further investigation.

2.4.4 Oxidative stress

As described above, the stringent response is involved in the formation of persisters by inducing antioxidant defenses and restricting the production of pro-oxidants (Nguyen *et al.*, 2011). In support of a role of oxidative stress in the formation of persisters, it was shown that H_2S induces antibiotic tolerance by mitigating oxidative stress via sequestration of ferrous iron and by stimulating activity of antioxidant enzymes (Shatalin *et al.*, 2011). Based on these results, it seems that active processes, like suppression of oxidative stress, are involved in persister formation, indicating that dormancy *per se* is not sufficient to explain the persistent phenotype. This is in accordance with other reports, where it was shown that the persistent state is far more complex than just a passive dormant state (see Section 2.2).

Oxidative stress and persistence are also correlated in species such as *E. coli* and *M. tuberculosis.* In general, lowered cellular levels of hydroxyl radicals counteract the bactericidal action of antibiotics in persister cells. These lowered levels can be reached by

increased levels of detoxifying enzymes. In *E. coli* and *S. aureus* it was shown that, as in *P. aeruginosa*, the production of H_2S alleviates oxidative stress by stimulating the activity of detoxifying enzymes. As a result, antibiotic tolerance increases (Shatalin *et al.*, 2011). In addition, another report showed that *M. tuberculosis* persister cells have a different susceptibility to OH• radicals produced during antibiotic treatment (Grant *et al.*, 2012). Furthermore, in *E. coli*, a mechanism was described in which cells increase their tolerance to quinolones in response to oxidative stress. Upon incubation of cells with a superoxide-generating agent, cells regulate expression of genes through SoxRS, including upregulation of an efflux pump which extrudes quinolones. The lower intracellular concentration of this antibiotic allowed a greater fraction of the population to become a persister cell (Wu *et al.*, 2012).

2.4.5 SOS-response

The SOS-response, which is activated upon DNA damage, has also been shown to play a role in *P. aeruginosa* persistence. A transposon mutant of *dinG*, encoding a putative DNA helicase, showed a decreased persister fraction upon antibiotic treatment (De Groote *et al.*, 2009). How exactly the SOS-response is involved in *P. aeruginosa* antibiotic tolerance is currently unknown. The SOS-response has been implicated in persistence of *E. coli*. It was shown that induction of the SOS-response activates the TisB toxin, part of the *tisAB* TA module (for information on TA modules see Section 2.4.3), which binds to the membrane and disrupts proton motive force, causing a drop in ATP levels. As a consequence, cellular targets of bactericidal agents are shut down, making the cell tolerant to antibiotic treatment (Dorr *et al.*, 2009; Dörr *et al.*, 2010).

2.5 Anti-persister strategies

As described in Section 2.3.2, persisters are a major cause of treatment failure in immunosuppressed or immunocompromised persons. The incomplete eradication of the infecting bacterial population gives rise to lingering infections, which negatively affects the patients' outcome. Therefore, it is important to find strategies to eliminate these antibiotic-tolerant cells. The first hypothetical solution to deal with persisters was already reported in 1944, along with the first description of these antibiotic-tolerant isolates in literature (1944). It

was suggested that treatment with a bactericidal agent which kills all susceptible cells, followed by withdrawal of the antibiotic, would allow persisters to resuscitate and regain susceptibility. By adding a second dose of antibiotics shortly after the persisters restarted growth, complete eradication of the bacterial population may be achieved. Many years later, this simple approach is supported by a mathematical model (De Leenheer & Cogan, 2009). Despite the successful *in vitro* result of such an approach (Lewis, 2007), the optimal *in vivo* protocol remains patient-specific and relies on bacterial kinetics, limiting the practical utility. Therefore, alternative approaches are needed to eliminate these antibiotic-insensitive cells.

In theory, different approaches can be pursued to eliminate persisters. The first one entails killing of persister cells either by designing an antibiotic capable of killing both the antibiotic-tolerant and -intolerant cells or by interfering with pathways essential for the persisters' dormant state. A second approach is represented by supplementation of conventional antibiotics with molecules that sensitize persisters to the action of these bactericidal agents. Alternatively, antibiotics can be combined with molecules that prevent or decrease the formation of persisters by interfering with processes implicated in persistence. Below, these options, together with some examples, are discussed in more detail.

2.5.1 Killing persisters

It is generally accepted that the antibiotic tolerant state of persisters results from their non-dividing, metabolically quiescent state, rendering the antibiotic targets inactive (Bigger, 1944; Balaban *et al.*, 2004; Keren *et al.*, 2004b; Shah *et al.*, 2006; Lewis, 2007; Wood *et al.*, 2013). However, integrity of the cell envelope remains crucial for viability, making these structures potential targets for anti-persister strategies. For instance, *E. coli* cells, including persisters, are prone to killing by membrane-acting peptides (Chen *et al.*, 2011). In addition, the artylysin Art-175, a bacteriophage-encoded endolysin coupled to a targeting peptide for guidance through the bacterial outer membrane, kills *P. aeruginosa* cells including persisters by puncturing the peptidoglycan layer (Briers *et al.*, 2014b).

More recent observations provide evidence that persister cells may use active processes to maintain their persistent state. For instance, it was shown that active suppression of the oxidative stress is involved in the formation of persisters (Nguyen *et al.*, 2011). As energy is needed to support these processes, an anti-persister approach may consist of inhibiting or

disturbing activity of respiratory membrane-associated enzymes (Hurdle *et al.*, 2011). An example of the latter is the diarylquinoline bedaquiline, acting on the membrane-associated ATP synthase in *M. tuberculosis* (Andries *et al.*, 2005). This compound is currently involved in Phase II clinical trials (NCT01691534 and NCT02193776). Another approach lies in targeting processes specifically needed to counteract stresses encountered in the host environment. For example, inhibitors of the *M. tuberculosis* dihydrolipoamide acyltransferase, an enzyme required to cope with nitric oxide-derived reactive nitrogen derivatives, proved to be almost exclusively bactericidal for non-multiplying *M. tuberculosis* cells (Bryk *et al.*, 2008). Identifying such processes in *P. aeruginosa* could provide a starting point for the rational design of an anti-persister therapy.

Another elegant strategy to kill persisters was published in which the specificity of a protease is removed by the acyldepsipeptide antibiotic ADEP4, resulting in self-digesting *S. aureus* cells (Conlon *et al.*, 2013). Upon addition of ADEP4, the protease ClpP no longer needs ATP to exert its action, explaining its activity in energy-deprived persister cells. Since resistance was rapidly acquired, ADEP4 is combined with a conventional antibiotic, leading to reduced accumulation of resistance. This combination led to complete eradication of *S. aureus* in a neutropenic mouse thigh infection model, simulating a chronic infection in immunocompromised persons, within 24 hours.

Since the physiological nature and mechanisms involved in persister formation and maintenance are not yet fully understood, it remains difficult to design a rational anti-persister strategy. Therefore, an alternative approach is to screen small-molecule collections to identify compounds that specifically kill dormant, non-replicating bacterial cells. A screening approach was described in which a nutrient-depleted stationary phase cell suspension of *S. aureus* was used to screen for novel drugs. This screen resulted in the identification of a quinolone-derived compound showing good activity against these non-replicating *S. aureus* cells both *in vitro* and in a mouse infection model. However, the drug was less potent on multiplying cells. The bactericidal activity of this compound results from destabilization of the cell membrane and destruction of the cell wall (Hu *et al.*, 2010). Additionally, a method was published to identify targets for the development of drugs that prevent bacterial persistence. This method is based on combined transcriptional and proteolytic silencing of a target gene and its resulting gene product, allowing identification of proteins essential during growth or non-growth (Kim *et al.*, 2013).

2.5.2 Sensitizing persister cells

Although persister cells perform translation at low levels (Shah *et al.*, 2006; Gefen *et al.*, 2008), they remain insensitive towards the bactericidal action of protein synthesis-inhibiting aminoglycoside antibiotics. This results from the low metabolic state of persisters, which, since the uptake of aminoglycosides is an energy-dependent process (Taber *et al.*, 1987), hampers entrance of these antibiotics into the bacterial cell. By adding certain metabolites to *S. aureus* or *E. coli* cultures, the generation of a proton-motive force increased the uptake of aminoglycosides and induced killing of bacterial cells (Allison *et al.*, 2011a). More recently, it was shown that this was also true for *P. aeruginosa* (Barraud *et al.*, 2013). In addition, it was shown that combining fluoroquinolones with phages expressing the major outer membrane protein OmpF, which forms porins that quinolones use to enter the bacterial cell (Nikaido, 2003), increased killing compared to monotreatment in *E. coli* (Lu & Collins, 2009).

Another example is provided by the synthetic QS inhibitor (Z)-4-bromo-5-(bromomethylene)-3-methylfuran-2(5H)-one (BF8), which has been described to revert multidrug tolerance in wild type (Pan *et al.*, 2012) and mucoid (Pan *et al.*, 2013a) *P. aeruginosa* strains. Examination of structural analogs of BF8 revealed that not all QS inhibitors were able to revert persistence, pointing to the existence of other targets in addition to the ones involved in QS (Pan & Ren, 2013). The exact mechanism by which this molecule sensitizes persister cells is currently unknown.

Lastly, screening of a small-molecule library identified a molecule that, in combination with ampicillin, significantly reduced the number of viable exponential phase cells compared to monotreatment with ampicillin (Kim *et al.*, 2011). This molecule, 3-[4-(4-methoxyphenyl) piperazin-1-yl] piperidin-4-yl biphenyl-4-carboxylate (C10), reverts *E. coli* persisters cells to growing, antibiotic-sensitive cells, although the exact mechanism is currently unknown. In addition, it was shown that C10, in combination with a fluoroquinolone antibiotic, reduced the level of persister cells of a *P. aeruginosa* culture (Kim *et al.*, 2011).

2.5.3 Interfering with processes implicated in *Pseudomonas aeruginosa* persistence

Processes shown to play a role in persistence provide excellent targets for anti-persister therapies. By modulating these processes, the formation or level of persisters can be decreased. Some examples are given below. However, since mechanisms of persister formation display a high degree of redundancy (see Section 2.4), additional actions may be needed to completely eradicate the persister population.

2.5.3.1 Interfering with stringent response

The stringent response plays an important role in *P. aeruginosa* persistence by influencing oxidative stress protection mechanisms as described in Section 2.4.4. Since mutant *P. aeruginosa* cells defective in the stringent response showed worse survival in a murine infection model (Nguyen *et al.*, 2011), components interfering with this stress response provide a good anti-persister strategy. Towards this end, inhibitors that prevent accumulation of the stringent response alarmone (p)ppGpp have been described. These molecules target RelA, which is absent in mammals, by interfering with binding of GDP/GTP to the active site (Wexselblatt *et al.*, 2010). More recently, a more potent inhibitor of RelA, named relacin, was reported to reduce the (p)ppGpp level in Gram-positives *in vivo* (Wexselblatt *et al.*, 2012) but failed to penetrate Gram-negatives efficiently.

2.5.3.2 Interfering with oxidative stress

Another possibility consists of interfering with oxidative stress responses. It was shown that killing of *E. coli* persister cells is enhanced when conventional antibiotics are combined with bacteriophages delivering SoxR, which is involved in the coordination of the cellular response to superoxide. By delivering SoxR to the bacterial cell, the response to superoxide stress is disturbed, enhancing the action of quinolones (Lu & Collins, 2009). Another way to enhance killing of persister cells is by stimulating production of intracellular reactive oxygen species induced by the action of antibiotics (Grant *et al.*, 2012; Brynildsen *et al.*, 2013).

2.5.3.3 Interfering with SOS-response

An additional stress response pathway that could be targeted is the SOS-response. Phages overexpressing the SOS-response repressor LexA3 significantly reduced persister levels in combination with different classes of antibiotics (Lu & Collins, 2009). Furthermore, infected mice showed a significantly increased survival rate upon combination treatment, pointing to the feasibility of such a combination strategy for clinical use. Interestingly, several RecA inhibitors have been identified which attenuate the SOS-response in *E. coli*, providing possible candidates to be used in combination with conventional antibiotics for complete eradication of bacterial infections (Wigle *et al.*, 2009).

2.5.3.4 Interfering with quorum sensing

Lastly, in theory, inhibitors of QS pathways should reduce the level of *P. aeruginosa* persister cells (see Section 2.4.2). Inhibitors of QS are described in Section 1.4.3).

2.6 Concluding remarks

With the many advances made in medical technology, the number and expected lifetime of patients with a suppressed or (partially) impaired immune system has increased. Here, persister cells may become a threat as they prevent total eradication of an infecting population upon antibiotic treatment. This small fraction of antibiotic-tolerant cells forms a reservoir of viable cells, capable of re-initiating an infection once the antibiotic pressure drops. In this way, the infection can become chronic.

Several anti-persister strategies have been proposed which rely on designing molecules that interfere with processes important in formation and/or maintenance of persister cells. However, the persistent state, as well as the mechanisms involved in formation of these antibiotic-tolerant cells are not yet clear. It also becomes clear that some processes implicated in persistence are bacterium-specific. To better understand the mechanistic basis of persistence in *P. aeruginosa*, further research is needed. In addition, it seems that multiple pathways play a role in the formation of persisters. This is illustrated by the fact that, to date, no mutant which completely lacks the formation of persister cells was reported. Therefore, rational design of an anti-persister therapy remains difficult. Furthermore, future experiments

need to reveal whether inhibiting one of these processes is sufficient to improve clearance of infection *in vivo*.

In the meantime, since no targets are available for rational design of an anti-persister therapy, a top-down approach can be used to identify molecules that, alone or in combination with a conventional antibiotic, kill the entire bacterial population including persister cells. The latter already proved to be successful (Kim *et al.*, 2011), however, this screening was carried out on growing cells, which most likely does not reflect the growth state *in vivo* were nutrients are limiting. Screening of compound libraries under test conditions which better reflect the *in vivo* environment may lead to potential anti-persister molecules.

Chapter 3

Scope of the thesis

This thesis focuses on the nosocomial pathogen *Pseudomonas aeruginosa*. As treatment options are limited, identification of new anti-pseudomonal agents as well as development of new treatment strategies, which eliminate infections more effectively, are necessary. A major factor responsible for treatment failure is the presence of persister cells. As they survive the bactericidal action of antibiotics, they form a reservoir of viable cells capable of causing relapse of infection. Therefore, targeting these persisters will greatly improve treatment. However, mechanisms underlying formation of *P. aeruginosa* persister cells remain largely unknown, making it hard to develop a rational anti-persister strategy. In order to obtain more insight into the mechanistic basis of persistence in *P. aeruginosa*, a screening was previously conducted to identify genes involved in fluoroquinolone tolerance in this pathogen (De Groote *et al.*, 2009). In this thesis, we follow up on this research. Additionally, a new research line was initiated, focusing on the development of an anti-persister therapy using small molecules.

In a first part of the thesis, we further characterize a newly identified gene, named *dnpA* (de-*N*-acetylase involved in persistence; gene locus PA14_66140/PA5002), which is a possible target for an anti-persister strategy. In **Chapter 4**, we demonstrate that this gene is a genuine persistence gene in *P. aeruginosa* and discuss how DnpA may contribute to fluoroquinolone tolerance. In **Chapter 5**, as a follow-up to this study, we determine the subcellular localization of DnpA in detail and examine its relation to persistence.

As it becomes clear that multiple mechanisms underlie the formation of persister cells, rational design of an anti-persister strategy is difficult. Therefore, in a second part of this thesis, we conducted a medium-throughput screening to identify small molecules that, in combination with conventional antibiotics, reduce the persister fraction of *P. aeruginosa*. **Chapter 6** describes the identification and initial characterization of such anti-persister

molecules. In **Chapter 7**, a more detailed characterization of one promising anti-persister molecule is carried out as well as first experiments to unravel its mode of action. These identified anti-persister molecules may serve as a starting point for the development of novel anti-persister therapies. Moreover, unraveling the mode of action of such molecules may reveal additional targets for the rational design of an anti-persister strategy.

Finally, **Chapter 8** contains the general conclusions of this thesis and some future perspectives.

As new anti-pseudomonal compounds are limiting, a screen for growth inhibitory compounds against *P. aeruginosa* was carried out in parallel with the one described in Chapter 6. Since this thesis focuses on persistence, the identification and initial characterization of a promising anti-pseudomonal compound is described in Addendum I.

Chapter 4

DnpA, a putative de-*N*-acetylase of the PIG-L superfamily affects fluoroquinolone tolerance in *Pseudomonas aeruginosa*[10]

4.1 Introduction

A major cause of antibiotic treatment failure is the presence of persister cells, phenotypic variants of the wild type that are transiently capable of surviving prolonged periods of antibiotic treatment. Upon a drop in antibiotic concentration, persisters can revert to normal cells and regrow into a susceptible population. A few years ago, a causal link between persister cells and recalcitrance of chronic infections was provided (Lafleur *et al.*, 2010; Mulcahy *et al.*, 2010) underlining the importance of this cell subpopulation in treatment failure.

Traditionally, persisters are considered to be non-dividing, metabolically quiescent cells in which inactivity of the antibacterial targets impairs the bactericidal effect (Bigger, 1944; Balaban *et al.*, 2004; Keren *et al.*, 2004b; Shah *et al.*, 2006; Lewis, 2007). Recently, evidence was provided that the persistent state is far more complex than just a passive dormant state. *Escherichia coli* persister cells display some degree of translation (Gefen *et al.*, 2008) and it was shown that a dormant state is not sufficient or required for a cell to become persistent (Orman & Brynildsen, 2013). In addition, no correlation between the growth rate of an individual cell and persistence was observed in *Mycobacterium* (Wakamoto *et al.*, 2013). The

[10] This Chapter was published as Liebens, V., Defraine, V., Van der Leyden, A., De Groote, V., Fierro Gutiérrez, A., Beullens, S., Verstraeten, N., Kint, C., Jans, A., Frangipani, E., Visca, P., Marchal, K., Versées, W., Fauvart, M., Michiels, J. (2014). A putative de-*N*-acetylase of the PIG-L superfamily affects fluoroquinolone tolerance in *Pseudomonas aeruginosa. Pathogens and Disease,* **71** (1), 39-54.
Analysis of transcriptome data described in this Chapter was performed by Dr. Carolina Fierro (KU Leuven)

molecular mechanism underlying persister formation in *Pseudomonas aeruginosa* is largely unknown and only few genetic determinants have been described to influence persister formation in this pathogen (Fauvart *et al.*, 2011). These include the quorum sensing determinants *lasI* and *lasR* (Kayama *et al.*, 2009), the alternative sigma factor RpoN (Viducic *et al.*, 2007) and the stationary phase regulators RpoS, DskA, RelA and SpoT (Murakami *et al.*, 2005; Viducic *et al.*, 2006). More recently, it was shown that persistence in *P. aeruginosa* is mediated by the stringent response. Activation upon starvation leads to suppression of oxidative stress by inducing antioxidant defenses and restricting the production of pro-oxidants (Nguyen *et al.*, 2011). This result further supports the theory that, as in *E. coli*, target inactivity *per se* is not sufficient to explain the persistent phenotype. In order to improve treatment of chronic *P. aeruginosa* infections, a better understanding of the persistent state and formation of these antibiotic-tolerant cells is necessary.

Previously, we performed a high-throughput screening of a *P. aeruginosa* PA14 mutant library to identify new persistence genes (De Groote *et al.*, 2009). In this Chapter, we demonstrate that *dnpA* (de-*N*-acetylase involved in persistence; gene locus PA14_66140/PA5002) is a genuine persistence gene in *P. aeruginosa* and discuss how DnpA may contribute to fluoroquinolone tolerance.

4.2 Experimental procedures

Bacteria and culture conditions. *P. aeruginosa* strains were cultured in Trypticase Soy Broth (TSB) or in Mueller Hinton Broth (MHB) at 37 °C. For solidified medium, 1.5 % agar was added. Following antibiotics were used: ofloxacin, ciprofloxacin, piperacillin, cefotaxime, ceftazdime, tobramycin, amikacin, polymyxin B, gentamicin (45 µg mL^{-1}), tetracycline (10 or 100 µg mL^{-1}) and ampicillin (100 µg mL^{-1}). Strains used in this study are listed in Table 4.1.

Persistence assay. The persistence assay was performed as described previously with minor modifications (De Groote *et al.*, 2009). Briefly, stationary phase cultures grown in MHB were treated with ofloxacin at a final concentration of 5 µg mL^{-1} for 5 hours at 37 °C while shaking at 200 rpm. A control treatment with sterile water was performed in parallel. The number of colony forming units (CFU) was determined by plate counts. The persister fraction is defined as the number of surviving cells after treatment with ofloxacin divided by the number of cells

after control treatment. These conditions were previously optimized as higher ofloxacin concentrations or increased incubation times do not lead to a further reduction of the number of surviving cells. Each experiment was independently repeated at least three times.

Table 4.1: Bacterial strains and plasmids used in this study

Strain	Description*	Source
Escherichia coli		
TOP10	F⁻ *mcr*A Δ(*mrr-hsdRMS-mcrBC*) φ80*lacZ*ΔM15 Δ*lacX* 74 *recA1* *araD139* Δ(*ara leu*) 7697 *galU galK*	Invitrogen
Pseudomonas aeruginosa		
PA14	Wild type	Liberati *et al.* (2006)
dnpA mutant	PA14 with an insertion in PA14_66140 *(dnpA)* (mutant ID 27910); Gm^R	Liberati *et al.* (2006)
PA14_66120	PA14 with an insertion in PA14_66120 (mutant ID 33516); Gm^R	Liberati *et al.* (2006)
Plasmids		
pME6032	pVS1 derived shuttle vector; Tc^R	Heeb *et al.* (2000)
pME6032-*dnpA*His	*dnpA* containing C-terminal His-tag cloned into pME6032	This chapter
pME6032-*dnpA*D190NHis	*dnpA*-D190N containing C-terminal His-tag cloned into pME6032	This chapter
pME6032-*dnpA*D340NHis	*dnpA*-D340N containing C-terminal His-tag cloned into pME6032	This chapter
pUC18	Broad host range shuttle vector; Ap^R	Yanisch-Perron *et al.* (1985)
pUC18-*dnpA*	EcoRI - PstI digestion of pME6032-*dnpA*His cloned into pUC18	This chapter

*Gm^R, gentamicin resistant; Tc^R, tetracycline resistant; Ap^R ampicillin resistant

MIC determination. The minimal inhibitory concentration (MIC) was determined with a broth microdilution procedure using MHB growth medium. A start inoculum of 5.10^5 cells per mL^{-1} was prepared and incubated in the presence of a 2-fold dilution series of each

antibiotic. After 24 hours of growth at 37 °C, the OD_{595} was measured and the lowest antibiotic concentration that completely inhibited bacterial growth was considered the MIC. The following concentration ranges were tested: ofloxacin (10 - 0.02 µg mL^{-1}), ciprofloxacin (2.5 - 0.005 µg mL^{-1}), piperacillin (60 - 0.10 µg mL^{-1}), cefotaxime (250 - 0.49 µg mL^{-1}), ceftazdime (100 - 0.2 µg mL^{-1}), tobramycin (8 - 0.016 µg mL^{-1}), amikacin (80 - 0.16 µg mL^{-1}) and polymyxin B (10 - 0.02 µg mL^{-1}). Each antibiotic was tested independently three times.

Biofilm assay. The assay was performed essentially as described previously (Merritt *et al.*, 2005; Maisonneuve *et al.*, 2013) with the following adjustments: *P. aeruginosa* stationary phase cultures were washed in phosphate buffered saline (PBS; composition for 1 L: 8 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4, 0.24 g KH_2PO_4, pH 7.4), diluted to an OD_{595} of 0.05 and spotted on sterile membranes (Durapore® Membrane Filter 0.22 µm GV, Millipore) placed on solid TSB. The plates were incubated at 37 °C for 24 hours. To assess the persister fraction, membranes were placed in parallel on solid medium with or without 20 µg mL^{-1} ofloxacin for 24 hours. Subsequently, the number of surviving cells was determined by resuspending the biofilm mass in sterile PBS by vigorous vortexing and plating serial dilutions on solid TSB medium. The number of persister cells was determined as described above.

Plasmid construction. For complementation experiments, *dnpA* was amplified by using primer couple SPI6628-6634. SPI6634 encodes a His-tag. The resulting PCR fragment was EcoRI - BglII cloned into pME6032 (Heeb *et al.*, 2000) and confirmed by sequencing. The plasmid was introduced into a *P. aeruginosa dnpA* insertion mutant by electroporation (Farinha & Kropinski, 1990). To obtain pUC18-*dnpA*, pME6032-*dnpA*His was digested with EcoRI - PstI and *dnpA* was subcloned into pUC18. For sequences of the primers see Table 4.2.

Lipopolysaccharide (LPS) analysis. LPS isolation was performed essentially as described previously (Hitchcock & Brown, 1983). Briefly, stationary phase cells were washed in PBS after which they were resuspended in 250 µL of Hitchcock and Brown lysis buffer. Samples were heated for 30 minutes at 100 °C, cooled to room temperature and incubated overnight at 55 °C with 1.5 µL proteinase K solution (20 µg mL^{-1}). For sodium dodecyl sulphate polyacrylamide gel electrophoresis analysis (SDS-PAGE), samples were heated at 100 °C for 5 minutes prior to loading on gel (NuPAGE® Novex, 12 % BisTris, Invitrogen). LPS profiles were visualised by the ultra-fast silver staining method described by Fomsgaard *et al*. (1990).

Table 4.2: Primers used in this Chapter

Primer name	Sequence
SPI6628	CACCGAATTCAGCATGAGCGCACGCAAG
SPI6634	ACTCAGATCTTCAATGATGATGATGATGATGCTCGAGCGCCTCCCCATCCAG
SPI5463	CATTTCGATCCGCACCCGAACCATGTCCGCGCCCAGG
SPI5464	CCTGGGCGCGGACATGGTTCGGGTGCGGATCGAAAT
SPI8820	TCGGCGTCGTTGGCATGCGGA
SPI8821	TCCGCATGCCAACGACGCCGA

Isolation of membrane fractions. Outer and inner membrane fractions were isolated by a modification of the Hancock and Nikaido method (Hancock & Nikaido, 1978). An overnight culture was diluted 100-fold into fresh medium. At an OD_{595} of 0.5, the cells were induced with 1 mM isopropyl ß-D -1-thiogalactopyranoside (IPTG) for one hour after which the cells were harvested by centrifugation. The resulting cell pellet was washed in a 30 mM Tris/HCl 150 mM NaCl buffer (pH = 7) and resuspended in a 30 mM Tris/HCl buffer (pH = 8) containing 20 % sucrose, 1 mM phenylmethylsulfonyl fluoride, 0.5 mg mL^{-1} lysozyme, 0.05 mg mL^{-1} DNase and 0.05 mg mL^{-1} RNase. After 30 minutes of incubation at 37 °C, the cells were further disrupted by sonication. The cell debris was removed by low speed centrifugation and the supernatant was layered onto a sucrose step gradient containing one mL of 70 % (wt/vol) sucrose and 6 mL of 30 % (wt/vol) sucrose in 30 mM Tris/HCl buffer (pH = 8). These samples were centrifuged at 183,000 ***g*** in a Beckman SW41Ti rotor for one hour. After centrifugation, the interface between the 30 % and 70 % layers was collected and used for another round of ultracentrifugation with a sucrose density gradient containing steps of one mL of 70 % (wt/vol) sucrose, 3 mL of 60 % (wt/vol) sucrose, 3 mL of 52 % (wt/ vol) sucrose, and 3 mL of 30 % (wt/vol) sucrose in Tris buffer. After overnight centrifugation (14 hours at 183,000 ***g***), the inner and outer membrane fractions were removed by suction from above. These fractions were washed in 20 % (wt/vol) sucrose (1 hour at 183,000 ***g***) and the resulting cell pellet was resuspended in a small volume of double distilled water. The outer membrane fraction was subjected to a treatment with 2 % *N*-lauryl-sarcosine (Sarcosyl) in order to ensure the purity of this fraction.

Site-directed mutagenesis. Site-specific mutations were introduced using the QuikChange Site-Directed Mutagenesis Kit (Stratagene) with pUC18-*dnpA* as template DNA. Briefly, a

PCR reaction was carried out using SPI5463-5464 and SPI8820-8821 for introduction of the D340N and D190N mutations, respectively, using a nonstrand-displacing DNA polymerase. The resulting PCR product was treated with DpnI to destroy the methylated parental non-mutated DNA after which it was transformed into chemocompetent *E. coli* TOP10 cells. Subsequently, *dnpA* was EcoRI-PstI subcloned into pME6032-*dnpA*His and the resulting plasmid was introduced into *P. aeruginosa* wild type by electroporation (Farinha & Kropinski, 1990). The presence of the correct mutation was verified by sequencing. Expression of mutant proteins was checked by western blotting. Sequences of the primers are listed in Table 4.2.

Western blotting. Overnight cultures were diluted 100-fold in TSB containing appropriate antibiotics and incubated at 37 °C while shaking at 200 rpm. After three hours of growth, cultures were induced with 1 mM IPTG for 4 hours after which the cells were collected by centrifugation and stored at -80 °C. The resulting cell pellet was resuspended in lysis buffer (8 M Urea, 100 mM NaH_2PO_4, 10 mM TrisCl, pH 8) containing 1 mg mL^{-1} lysozyme and incubated at 37 °C for 30 minutes. Subsequently, the cells were further disrupted by sonication after which the total protein fraction was separated from the cell debris by centrifugation. Next, proteins were separated by SDS-PAGE on a NuPAGE® Novex 12 % BisTris protein gel (Invitrogen). Following western blotting, hybridization was performed using a monoclonal anti-His_6 antibody solution (Roche) and Anti-Mouse IgG Alkaline Phosphatase Conjugated Antibody (Sigma-Aldrich) as primary and secondary antibody, respectively.

Sample preparation and microarray hybridization. Overnight cultures were diluted 100-fold into fresh medium and grown until late-exponential phase (OD_{595} = 1). The RNA content of 20 mL bacterial culture was stabilized by adding 1:5 volume of ice-cold phenol:ethanol (5:95) after which cells were harvested by centrifugation. The cell pellet was frozen in liquid nitrogen and stored at -80 °C. Total RNA was isolated as described by Vercruysse *et al.* (2010) and Meysman *et al.* (2014) using the Pure Link RNA mini Kit (Ambion). RNA integrity was evaluated using Experion RNA StdSens Chips (Bio-Rad). RNA quantity and purity was assessed by measuring the A260/A280 and A260/A230 ratio of each sample. The ratio of all samples was ≥ 1.8. For each strain RNA isolation was done in triplicate. cDNA generation, fragmentation, biotinylation and hybridization on GeneChip *P. aeruginosa*

Genome Arrays (Affymetrix) was performed according to the Affymetrix protocol by The Centre for Applied Genomics, The Hospital for Sick Children, Toronto, Canada.

Microarray data analysis. Microarray data were normalized using RMA implemented in the Bioconductor "affy" package. Pearson correlation across samples and spectral map analysis (Wouters *et al.*, 2003) were used to check consistency between samples and to identify an outlier that was removed from subsequent analysis. Differential expression was calculated using an empirical Bayesian method implemented in the LIMMA R package (Smyth, 2004). P-values were adjusted for multiple testing using the Benjamini & Hochberg method (Benjamini & Hochberg, 1995). A final list of differentially expressed genes was obtained at a False Discovery Rate (FDR) of 15 %. Lower FDR thresholds drastically reduced the gene selection and resulted in very limited functional annotation. *P. aeruginosa* gene annotation was obtained from www.pseudomonas.com (Winsor *et al.*, 2011) and KEGG (Kanehisa & Goto, 2000). PseudoCAP functional classes were used to calculate functional enrichment on the lists of differentially expressed genes. Enriched terms were obtained with Fisher's exact test at a FDR of 5 %.

Network analysis. *P. aeruginosa* functional interactions were obtained from the STRING database (Jensen *et al.*, 2009). Only gene-gene interactions with a minimum reliability score of 0.8 were retained. A deregulated network was extracted by selecting interactions in which both genes are differentially expressed in the *dnpA* mutant and/or under *dnpA* overexpression. Network visualization was done with Cytoscape (Smoot *et al.*, 2011). The deregulated network was used to identify groups of genes which are consistently up/down (or down/up) regulated in the *dnpA* mutant and in the strain overexpressing *dnpA*, respectively. Two community detection methods were used to subdivide the largest network component into smaller sub-networks: walktrap (Pons & Matthieu, 2005) and edge betweenness (Newman & Girvan, 2004) algorithms. The 58 smallest network components were used as independent sub-networks without any modification. We used Fisher's exact test to select sub-networks with significant and consistent differential expression ($P < 0.05$). Only the genes present in the deregulated network were considered for Fisher's exact test. Each significant and consistent sub-network was annotated with the PseudoCAP functional classes (see above).

Statistical analysis. For each comparative analysis, significant difference in variance of the log persister fractions was checked using a Fisher F-test. In case of statistically different

variances a Welch test for the means was applied, otherwise a student's t-test was performed. Normality of the log persister fractions was verified.

4.3 Results

4.3.1 DnpA is involved in fluoroquinolone tolerance

Previously, a *P. aeruginosa* PA14 random insertion mutant library was screened to identify new persistence genes. Nine mutants with an altered persister fraction after treatment with the fluoroquinolone antibiotic ofloxacin were selected (De Groote *et al.*, 2009). One of these mutants, CMPG13401, displays a 10-fold decreased persister fraction compared to the wild type and was selected for further characterization. The mutant contains an insertion in gene locus PA14_66140 (PA5002 in *P. aeruginosa* PAO1, Figure 4.1) which we propose to rename *dnpA* (de-*N*-acetylase involved in persistence). The mutant displays the same MIC value for ofloxacin as the isogenic wild-type strain (De Groote *et al.*, 2009), excluding the involvement of fluoroquinolone resistance mechanisms. Growth characteristics of the mutant were indistinguishable from wild-type growth (De Groote *et al.*, 2009).

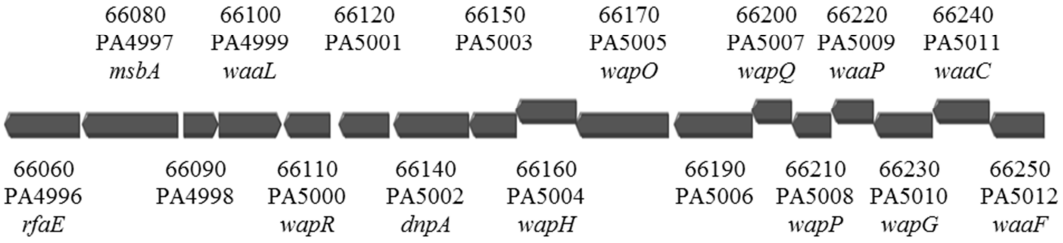


Figure 4.1: Overview of LPS core oligosaccharide gene cluster. Arrows indicate ORFs, gene names and both PAO1 and PA14 gene numbers are indicated.

Next, we analyzed a mutant in the same gene obtained from an independent mutant library (Liberati *et al.*, 2006). Similarly, the strain shows a 6-fold ($P < 0.01$) decrease in persister cells (Figure 4.2A), while no differences could be detected in MIC (Table 4.3) and growth characteristics (data not shown). All subsequent experiments were carried out with this strain. Controlled overexpression of *dnpA* in the *dnpA* mutant resulted in a 2.6-fold increase of the persister fraction compared to the empty vector control (Figure 4.2B). It should be noted that

the persister fraction of wild type and *dnpA* mutant strain containing the empty vector is similar. Finally, overexpression of the *dnpA* allele in the wild-type background increased the persister fraction 4-fold ($P < 0.05$; Figure 4.2C). Taken together, these results strongly support a role for *dnpA* in fluoroquinolone tolerance in *P. aeruginosa*.

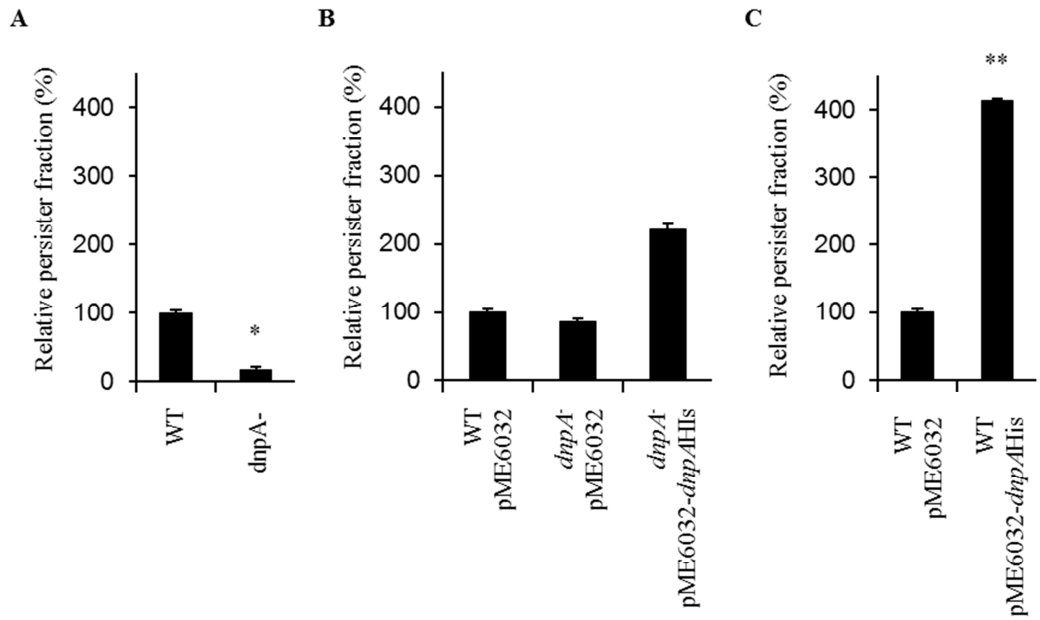


Figure 4.2: Relative persister fractions. Stationary phase cells of **A.** wild type (WT) and *dnpA* mutant (*dnpA*$^-$) **B.** wild type and *dnpA* mutant containing plasmid pME6032 (WT\pME6032 and *dnpA*$^-$\pME6032 respectively) and *dnpA* mutant overexpressing *dnpA* from the plasmid pME6032 (*dnpA*$^-$\pME6032-*dnpA*His) **C.** wild type carrying pME6032 and wild type strain overexpressing *dnpA* from the plasmid pME6032 (WT\pME6032 and WT\pME6032-*dnpA*His respectively) were treated in parallel with ofloxacin and sterile water for 5 hours. Subsequently, the cell suspension was diluted and plated out after which the number of surviving cells was determined. Data points correspond to the mean relative persister fraction. Each experiment was repeated independently at least three times. Error bars represent the standard error of the mean (SEM). A persister fraction of 100 % corresponds to 1.04×10^{-4} (panel A) and 3.43×10^{-5} (panel B and C). *$P < 0.01$, **$P < 0.05$.

Polar effects on expression of genes located downstream of the transposon insertion site in *dnpA* could contribute to a change in fluoroquinolone tolerance of the *dnpA* mutant. However, recent RNA-Seq data suggest that *dnpA* (PA14_66140) is the distal gene of a transcriptional unit starting from PA14_66170 (Wurtzel *et al.*, 2012) or PA14_66150 (Filiatrault *et al.*, 2010; Dotsch *et al.*, 2012), rendering this possibility unlikely. To formally exclude polar effects, a mutant with an insertion in the downstream gene PA14_66120 (Liberati *et al.*, 2006) was

analyzed. The mutant displays wild-type levels of fluoroquinolone tolerance (with an absolute persister fraction of $1.04 \times 10^{-4} \pm 3.81 \times 10^{-6}$ and $2.86 \times 10^{-4} \pm 1.99 \times 10^{-5}$; P-value = 0.12 for wild type and PA14_66120 mutant, respectively) suggesting that PA14_66120 is not required for persistence. More importantly, this result shows that the decrease in persistence observed for the *dnpA* insertion mutant is solely caused by inactivation of *dnpA*.

4.3.2 DnpA is involved in biofilm persistence

Persisters are believed to contribute significantly to the recalcitrance of biofilm-associated infections (Lewis, 2005; Percival *et al.*, 2011). To analyse whether *dnpA* is required for persistence in biofilms, we performed a recently described colony biofilm susceptibility assay (Maisonneuve *et al.*, 2013). Static biofilms were grown on a membrane during 24 hours after which they were exposed to ofloxacin for 24 hours. As shown in Figure 4.3A, the *dnpA* mutant exhibits a 10-fold reduction in persister fraction ($P < 0.1$). Biofilm formation of the mutant strain was not different from the wild type as determined by viable cell counts of untreated biofilms (Figure 4.3B). These results show that *dnpA* plays a role in persistence both in free-living conditions and in a biofilm mode of growth.

4.3.3 The antibiotic resistance profile of the *dnpA* mutant is unchanged

The two gene loci adjacent to *dnpA* were previously implicated in antibiotic resistance. The PA14 derivatives mutated in PA14_66120 and PA14_6150, respectively downstream and upstream of *dnpA*, were identified in several independent large-scale, systematic screenings for resistance genes. Both genes are involved in susceptibility to the penicillin antibiotic piperacillin, the cephalosporines ceftazidime and cefotaxime, and the carbapenem meropenem (Wiegand *et al.*, 2008; Dotsch *et al.*, 2009; Alvarez-Ortega *et al.*, 2010). In addition, it was shown that PA14_66120 is also involved in susceptibility to the aminoglycoside tobramycin (Schurek *et al.*, 2008). However, neither mutant was implicated in resistance to fluoroquinolone antibiotics (Breidenstein *et al.*, 2008; Dotsch *et al.*, 2009). We therefore decided to test the sensitivity of the *dnpA* mutant to antibiotics belonging to different classes: piperacillin (penicillins), cefotaxime and ceftazidime (cephalosporins), ciprofloxacin and ofloxacin (fluoruoquinolones), tobramycin and amikacin (aminoglycosides) and polymyxin B. The MIC values for PA14 wild type and *dnpA* mutant are listed in Table 4.3. The mutant

displays unaltered susceptibility towards all antibiotics listed. Modest changes in MIC value (2-fold) were observed for the β-lactams piperacillin and cefotaxime but such changes are generally considered within the acceptable range of error.

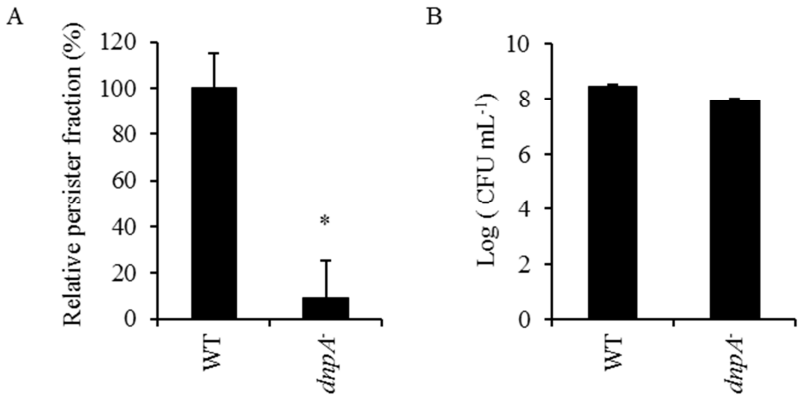


Figure 4.3: DnpA is involved in biofilm persistence. A. Biofilms formed by wild type (WT) and *dnpA* mutant (*dnpA*$^-$) strains were subjected to a 24 hour treatment. Subsequently, the cells were diluted and plated out after which the number of surviving cells was determined. Data points correspond to the mean relative persister fraction. A persister fraction of 100 % corresponds to 3.22×10^{-3}. *P < 0.1. **B.** The number of viable cells within untreated biofilms of WT and *dnpA*$^-$. Data points correspond to the mean. Each experiment was repeated independently at least three times. Error bars represent SEM.

Table 4.3: Antibiotic resistance profile of wild type and *dnpA* mutant. MIC (μg mL^{-1}) for each antibiotic is shown.

Antibiotic	Wild type	*dnpA* mutant
Ofloxacin	1.25	1.25
Ciprofloxacin	0.15	0.15
Piperacillin	15	30
Cefotaxime	31.25	62.50
Ceftazidime	6.25	6.25
Polymyxin B	0.32	0.32
Tobramycin	0.50	0.50
Amikacin	1.25	1.25

4.3.4 Genomic conservation of DnpA

dnpA is part of the LPS biosynthesis gene cluster that comprises *P. aeruginosa* PA14 genes PA14_66250 to PA14_66060. Conservation analysis revealed that the cluster is present in

each of the 45 sequenced *Pseudomonas* genomes (totaling 13 species)(Winsor *et al.*, 2011), as well as in *Azotobacter vinelandii*, a close relative of *Pseudomonas* (Rediers *et al.*, 2004)(Figure 4.4). Surprisingly, an *dnpA* orthologue was also identified in the genome sequence of the unclassified β-proteobacterium "*Candidatus* Accumulibacter phosphatis". The first seven genes of this cluster (PA14_66250-PA14_66190) are well conserved in all sequenced *Pseudomonas* species with regard to their presence and location within the cluster and are involved in the inner core biosynthesis of the LPS oligosaccharide (King *et al.*, 2009). Even though most of the other cluster genes are present throughout all of the sequenced *Pseudomonas* species (except for *wapH*, *wapR*, PA14_66120, *waaL* and PA14_66060), our analysis revealed that they exhibit a low level of synteny. PA14_66150-*dnpA* is conserved as one unit in all the analyzed genomes; however, its genomic location relative to the cluster is not conserved. This is most pronounced in *Pseudomonas putida* where they are clustered in a different genomic location. This might implicate that these genes are not necessarily involved in the LPS core oligosaccharide synthesis. PA14_66150 is related to the *Salmonella enterica* sv. Typhimurium regulatory *mig-14* gene (Pfam PF07395). Recently, it was shown that this locus is involved in lipid A modification upon recognition of cationic antimicrobial peptides (AMPs) in *P. aeruginosa* PAO1 (Jochumsen *et al.*, 2011). The glycosyltransferase WapR is the least conserved locus in the cluster.

4.3.5 DnpA does not cause major LPS modifications

To assess whether DnpA is involved in LPS biosynthesis or modification, LPS profiles of wild type, *dnpA* mutant and *dnpA* overexpressing strains were compared (Figure 4.5A). No differences in LPS profile were visible between these strains and their respective wild-type parent. This result indicates that DnpA, if involved in LPS synthesis, likely induces minor changes. These minor changes do not disrupt LPS core stability and therefore do not result in altered LPS profiles. In addition, we compared outer and inner membrane protein profiles of wild type and *dnpA* mutant but no significant differences could be detected (Figure 4.5B).

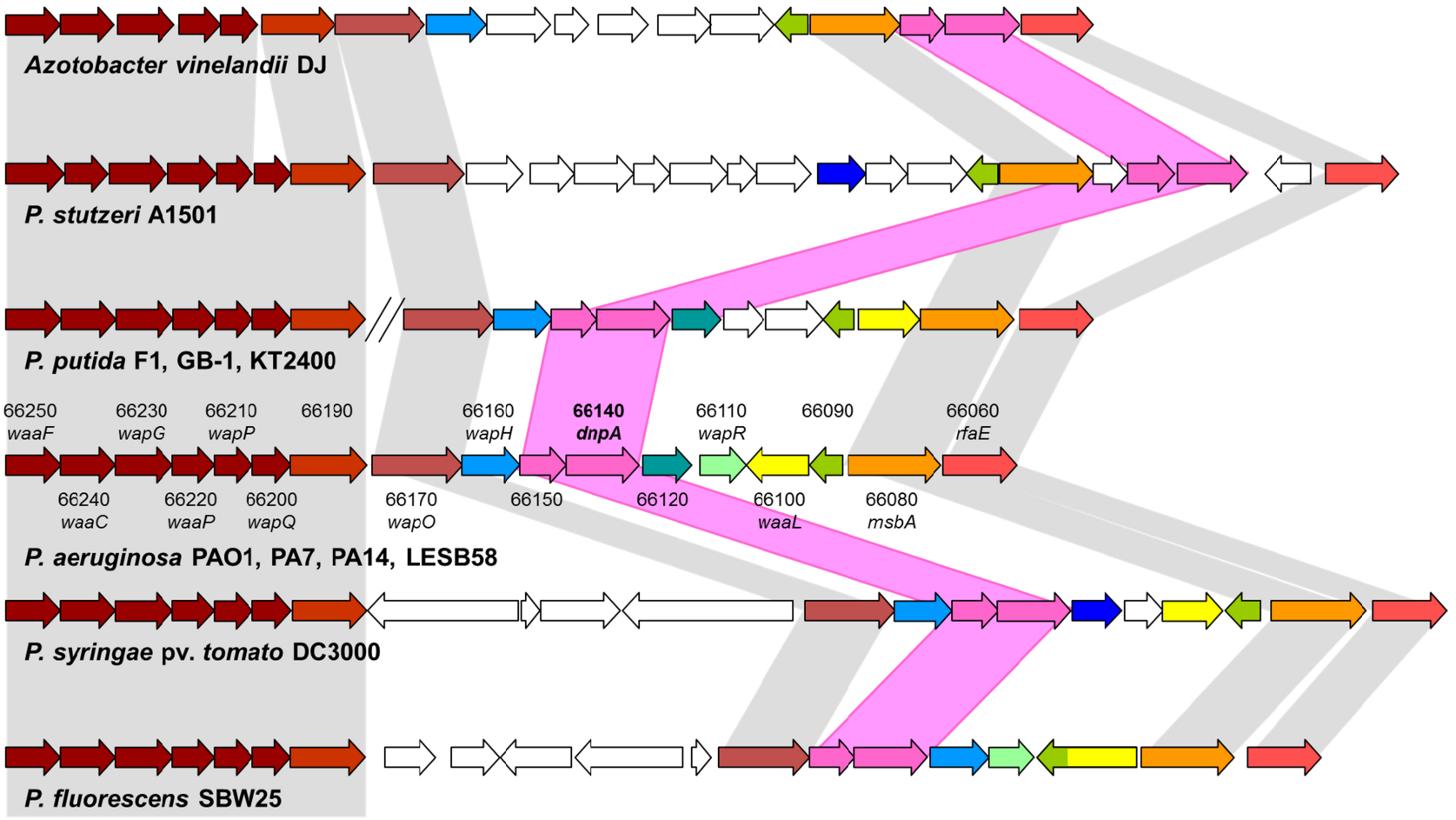


Figure 4.4: Conservation and synteny of the LPS core oligosaccharide gene cluster. A representative set of genomes of sequenced *Pseudomonas* species and the closely related *Azotobacter vinelandii* are shown. Arrows indicate Open Reading Frames (ORFs), coloured arrows indicate genes that correspond to the PA14 cluster genes. Orthologous ORFs are shaded in identical colors and are connected by grey areas, pink areas connect orthologues of the PA14_66150-*dnpA* unit. // indicates discontinuity in gene order. Gene names and PA14 gene numbers are indicated.

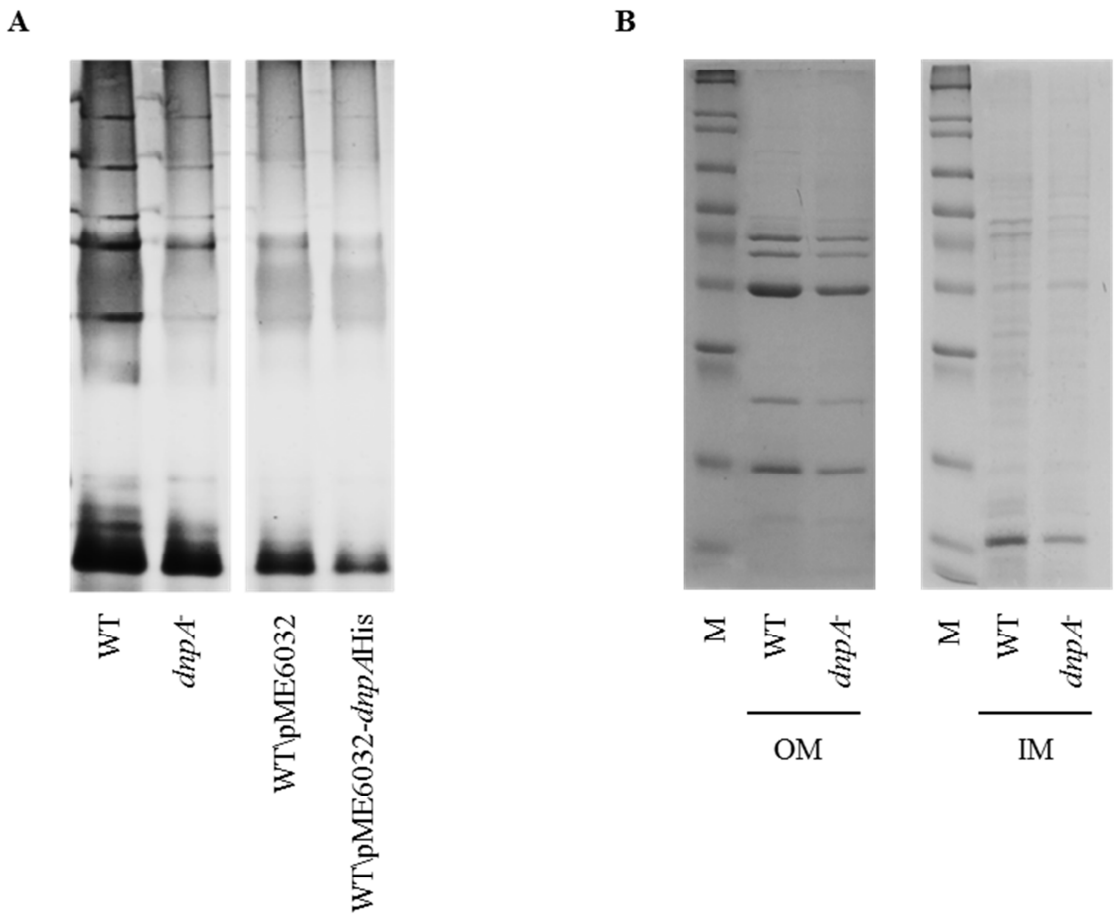


Figure 4.5: LPS profiles and membrane fractions. A. LPS profiles of wild type, *dnpA* mutant and *dnpA* overexpressing strains. LPS was isolated and analyzed as described in material and methods. LPS profiles of WT and *dnpA* mutant (*dnpA*$^-$) (left panel) and of WT containing plasmid pME6032 (WT\pME6032) and WT overexpressing *dnpA* from the plasmid pME6032 (WT\pME6032-*dnpA*His) (right panel). **B.** SDS-PAGE analysis of outer (OM) and inner membrane (IM) fractions of wild type and *dnpA* mutant (*dnpA*$^-$) respectively. Membrane fractions were isolated as described in material and methods. M: Wide range SigmaMarker (Sigma-Aldrich).

4.3.6 DnpA is a putative de-*N*-acetylase

Based on its deduced amino acid sequence DnpA is a member of the PIG-L or LmbE-like superfamily (Pfam PF02585) grouping over 4150 protein sequences (as of January 2014) from eukaryotes, archaea and bacteria. Over a dozen of these have been biochemically characterized and were shown to deacetylate the *N*-acetyl group of *N*-acetylglucosamine in various substrates (Watanabe *et al.*, 1999; Newton *et al.*, 2000; Chang *et al.*, 2002; Smith *et al.*, 2002; Steffek *et al.*, 2003; Tanaka *et al.*, 2004; Urbaniak *et al.*, 2005; Vats *et al.*, 2005; Ho *et al.*, 2006; Truman *et al.*, 2007; Yokoyama *et al.*, 2008; Wehmeier & Piepersberg, 2009;

Deli *et al.*, 2010; Gaballa *et al.*, 2010; Ashraf *et al.*, 2013). The proteins typically display low overall sequence identity (~25 %) but share a catalytic site containing a number of conserved residues that are essential for enzymatic activity. Multiple sequence alignment of DnpA with all PF02585 members with reported activity suggests that crucial amino acid residues of the DnpA active site are intact (Figure 4.6). We therefore predict that DnpA is also a de-*N*-acetylase, probably acting on *N*-acetylglucosamine residues, though the exact nature of its substrate remains to be determined.

Homo sapiens PIG-L	A	H	P	D	D	E	53	G	H	S	N	H	I	158
Rattus norvegicus PIG-L	A	H	P	D	D	E	53	G	H	S	N	H	I	158
Trypanosoma brucei TbGPI12	A	H	P	D	D	E	45	S	H	A	N	H	V	151
Leishmania major LmGPI12	A	H	P	D	D	E	45	G	H	P	N	H	I	151
Plasmodium falciparum	A	H	P	D	D	E	61	G	H	P	N	H	I	167
Entamoeba histolytica	A	H	A	D	D	D	47	G	H	P	N	H	I	144
Thermococcus kodakaerensis Tk-Dac	P	H	P	D	D	C	44	G	H	P	D	H	R	152
Nonomuraea sp. Dbv21	P	H	L	D	D	A	20	K	H	P	D	H	K	162
Actinoplanes teichomyceticus Orf2*	P	H	L	D	D	A	20	E	H	P	D	H	E	165
Streptomyces fradiae NeoL	P	H	P	D	D	V	35	G	H	V	D	H	L	158
Streptomyces kanamyceticus KanN	P	H	P	D	D	I	13	G	H	V	D	H	R	137
Mycobacterium tuberculosis MshB	A	H	P	D	D	E	17	G	H	P	D	H	V	148
Mycobacterium tuberculosis Mca	A	H	P	D	D	E	16	P	H	P	D	H	I	143
Bacillus anthracis MshB1	A	H	A	D	D	V	16	R	H	P	D	H	A	114
Bacillus circulans BtrD	P	H	F	D	D	V	18	K	H	V	D	H	R	167
Bacillus cereus BcZBP	A	H	A	D	D	V	16	R	H	P	D	H	A	114
Pseudomonas aeruginosa DnpA	P	H	A	D	D	A	192	P	H	P	D	H	V	342
		◆		★	◆				●			◆		

Figure 4.6: Partial sequence alignment of the active site of DnpA and related *N*-deacetylases. All proteins except for DnpA were previously demonstrated to exhibit enzymatic activity (see indicated references below). Fully conserved residues are shown in white text on a black background. Metal binding residues are indicated by diamonds, the catalytic base by a star and the substrate binding residue by a circle. The alignment was constructed using Cobalt (Papadopoulos & Agarwala, 2007). Full species names and Genbank protein accession numbers: *Homo sapiens*, BAA74775 (Watanabe *et al.*, 1999); *Rattus norvegicus*, BAA74776 (Watanabe *et al.*, 1999; Urbaniak *et al.*, 2005); *Trypanosoma brucei*, AAN60997 (Chang *et al.*, 2002); *Leishmania major*, AAN60998 (Chang *et al.*, 2002); *Plasmodium falciparum*, CAG25059.2 (Smith *et al.*, 2002); *Entamoeba histolytica*, XP_654497.1 (Vats *et al.*, 2005; Ashraf *et al.*, 2013); *Thermococcus kodakaraensis*, Q6F4N1 (Tanaka *et al.*, 2004); *Nonomuraea* sp. ATCC39727, CAD91216 (Ho *et al.*, 2006); *Actinoplanes teichomyceticus*, CAG15014 (Ho *et al.*, 2006); *Streptomyces fradiae*, BAD95829 (Yokoyama *et al.*, 2008); *Streptomyces kanamyceticus*, CAF31592 (Wehmeier & Piepersberg, 2009); *Mycobacterium tuberculosis*, NP_215686 (Newton *et al.*, 2000) and NP_215598 (Steffek *et al.*, 2003); *Bacillus anthracis*, AAP25493 (Gaballa *et al.*, 2010); *Bacillus circulans*, BAE07068 (Truman *et al.*, 2007); *Bacillus cereus*, NP_831313 (Deli *et al.*, 2010); *P. aeruginosa*, NP_253689.

Overexpression of *dnpA* in a wild-type background increases the persister fraction significantly. To test if this putative de-*N*-acetylase activity is important for persistence, two

specific point mutations were independently introduced into the putative catalytic site of DnpA. Expression of the mutant alleles was verified by western blotting (Figure 4.7A). Next, the effect of overexpression of the mutant alleles on persistence was examined. The first mutation converts Asp-340 into Asn (D340N). This specific mutation has been reported to inactivate the *in vitro* de-*N*-acetylase activity of the *Bacillus cereus* PIG-L protein BC1534 on many but not all substrates (Deli *et al.*, 2010). Secondly, a D190N mutation was introduced, changing the predicted catalytic base Asp-190 into Asn. The corresponding mutation in mammalian PIG-L completely abolishes de-*N*-acetylase activity (Urbaniak *et al.*, 2005). The persister fraction in a wild-type background increased significantly upon overexpression of DnpA-D190N ($P < 0.05$), whereas overexpression of the DnpA-D340N allele had no effect (Figure 4.7B).

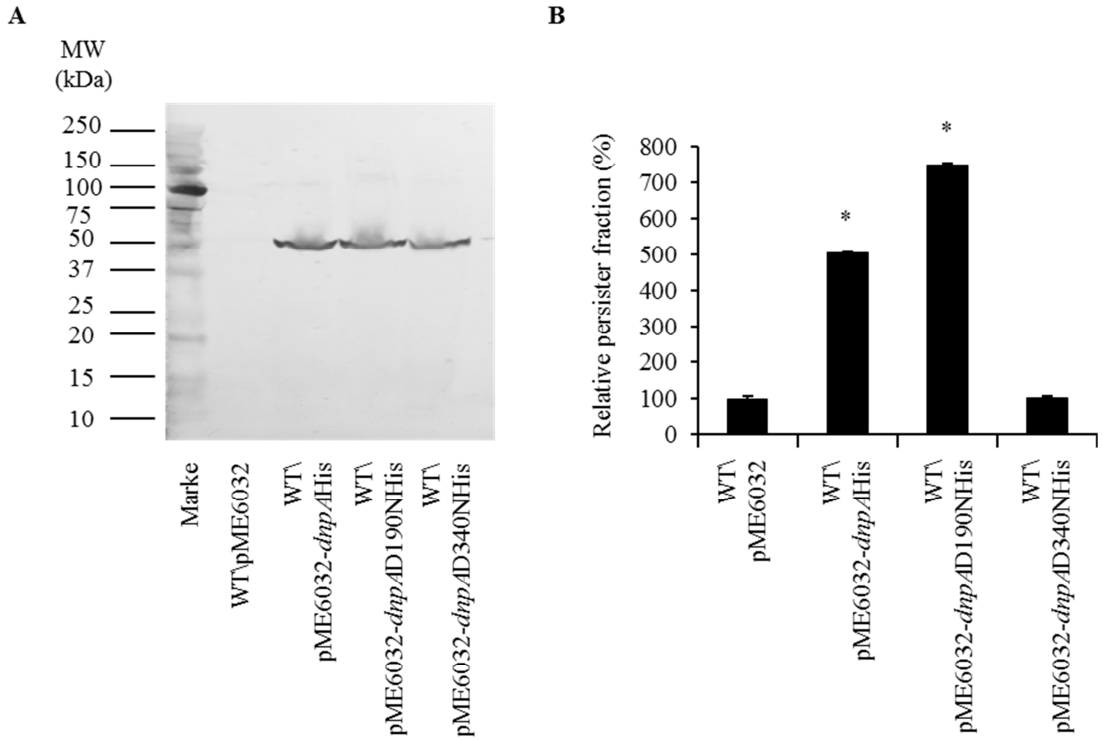


Figure 4.7: A. Western blot analysis of cells expressing wild-type and mutant *dnpA* alleles from pME60332. Precision Plus Protein Kaleidoscope Standards (Bio-Rad) was used as molecular marker. The molecular weight of DnpA-His is approximately 54,64 kDa. **B.** Relative persister fraction upon overexpression of wild-type and mutant *dnpA* alleles. The persister fraction was determined by treating stationary phase cells for 5 h with ofloxacin and sterile water respectively. Prior to treatment, cells were induced with IPTG to ensure expression of *dnpA* from the Ptac promoter on pME6032. Data points correspond to the mean relative persister fraction. Each experiment was repeated independently at least three times. Error bars represent SEM. A relative persister fraction of 100 % corresponds to 4.59×10^{-5}. $P < 0.05$.

4.3.7 Trancriptome analysis

In order to shed more light on the cellular function of DnpA and its role in fluoroquinolone tolerance in *P. aeruginosa*, a transcriptome analysis was carried out. Sampling was carried out at late-exponential phase, when the persister fraction started to increase (Figure 4.8). RNA from PA14 wild type, *dnpA* mutant and PA14 wild type overexpressing *dnpA* was isolated in triplicate and analyzed on GeneChip® *P. aeruginosa* Genome Arrays (Affymetrix). Differentially expressed genes were identified as described in Experimental procedures.

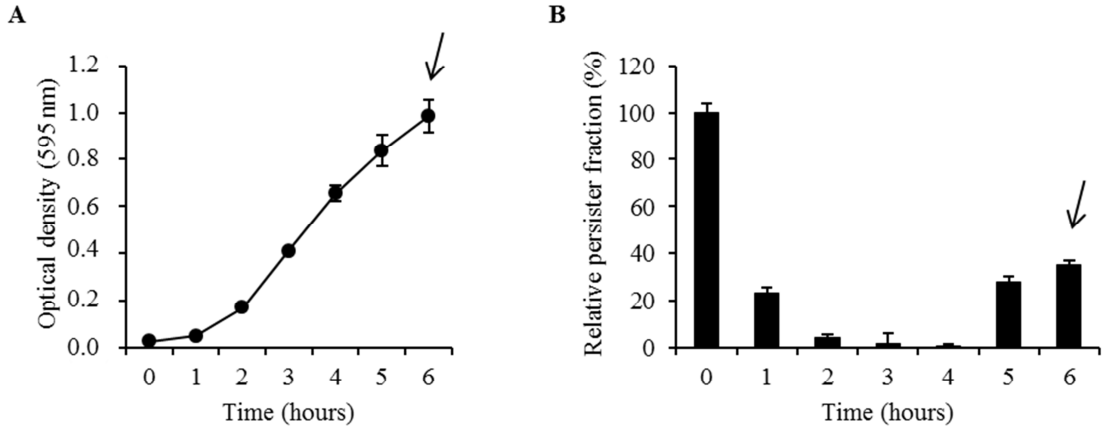


Figure 4.8: Evolution of the persister fraction after dilution into fresh medium. Stationary phase cells were diluted 100-fold into fresh medium. Every hour, an aliquot was taken of which **A.** the optical density (595 m) and **B.** the persister fraction was monitored. The persister fraction was determined following a 5 h treatment with ofloxacin. Untreated cells were used as a control. Data points correspond to the mean relative persister fraction. Each experiment was repeated independently at least three times. Error bars represent SEM. A relative persister fraction of 100 % corresponds 2.15×10^{-5}. Samples for RNA isolation were taken at an OD_{595} of 1 when the persister fraction started to increase (indicated by an arrow).

In total, 601 and 393 genes were differentially expressed in mutant and overexpression strain, respectively. In the mutant, 54.1 % (327/601) of the genes were downregulated whereas in the overexpression strain this percentage increased up to 63.9 % (251/393) (Figure 4.9).

The differentially expressed genes were classified into functional categories according to the PseudoCAP categories for both *dnpA* mutant and overexpression strain. Enriched functional categories were obtained according to Fisher's exact test ($P < 0.05$). This resulted in five enriched functional categories for both *dnpA* mutant and overexpression strain (Figure 4.10), of which categories "Amino acid biosynthesis and metabolism" and "Energy metabolism"

were shared among both conditions. For all categories, the majority of the genes was downregulated.

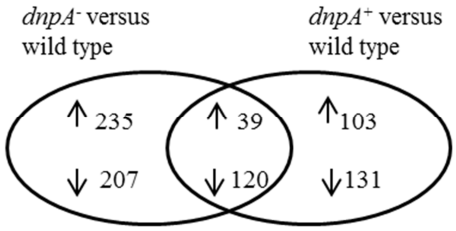


Figure 4.9: Gene expression overview. Venn diagram representing the number of differentially expressed genes in the *dnpA* mutant (*dnpA*⁻) and the *dnpA* overexpression strain (*dnpA*⁺) and the overlap between the two strains. Upward- and downward-oriented arrows indicate gene induction and repression, respectively.

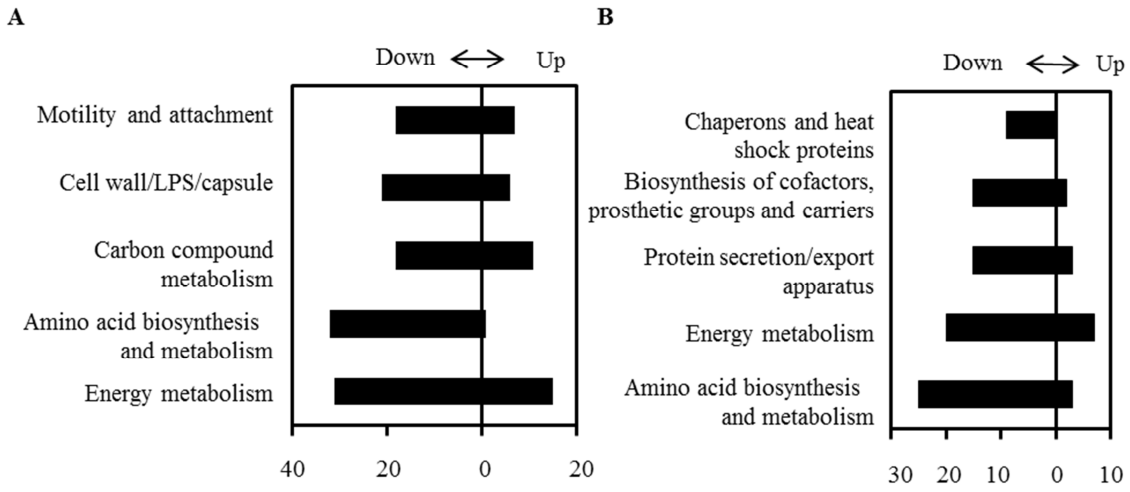


Figure 4.10: Differentially expressed genes grouped by functional categories. The number of up-and downregulated genes are shown for each functional category. The differential gene expression of **A.** the *dnpA* mutant and **B.** the *dnpA* overexpression strain using wild type as a reference condition.

4.3.8 Functional network analysis of differentially expressed genes

To identify processes that are consistently affected in both the *dnpA* mutant and overexpression strain, a functional interaction network was constructed. This network was constructed based on interactions in which both genes were differentially expressed in the *dnpA* mutant and/or *dnpA* overexpression strain. This deregulated network contains 429 genes and 1030 interactions and resulted in a large network component of 253 genes and 850

interactions and 58 smaller components with 2 to 16 genes. Based on this network we selected 5 sub-networks that were consistently up/down or down/up in both conditions (Fisher's exact test $P < 0.05$). These sub-networks were annotated with the PseudoCAP functional classes and are shown in Figure 4.11. Genes belonging to each sub-network are shown in Table 4.4.

Sub-network 1 contains genes with a role in amino acid biosynthesis and metabolism. Genes of this category that are differentially expressed in the *dnpA* overexpressing strain are downregulated. Likewise, genes of sub-network 4 belonging to the amino acid biosynthesis and metabolism functional category are downregulated in the *dnpA* overexpressing strain. In addition, genes with a role in energy metabolism (sub-network 5) are downregulated upon overexpression of *dnpA*. Taken together, these results suggest that overexpression of *dnpA* may lead to a lower metabolic activity.

Sub-network 2 consists of genes all classified into the "Cell wall/LPS/Capsule" functional category. Overall, these genes are downregulated in the *dnpA* mutant. However, as mentioned earlier, no difference could be detected between LPS profiles of wild type and mutant. Genes belonging to the third sub-network are all involved in motility and attachment. More specifically, genes involved in synthesis of type IV pili are downregulated in the mutant while a regulator of twitching motility *fimS/algZ* (Whitchurch *et al.*, 1996) is upregulated in the overexpressing strain.

4.4 Discussion

We report the characterization of a new *P. aeruginosa* persistence gene named *dnpA* (PA14 locus PA14_66140/PAO1 locus PA5002). A *dnpA* insertion mutant shows a reduced persister fraction both in broth and in a colony biofilm model while overexpression of the *dnpA* allele in the wild-type background increases the persister fraction. Furthermore, the corresponding PAO1 mutant displays a similar decrease in persister fraction (De Groote *et al.*, 2009) lending support for a non-strain-specific role of *dnpA* in fluoroquinolone tolerance.

dnpA is part of a gene cluster responsible for synthesis of the LPS core that contains 17 ORFs (*P. aeruginosa* PA14 PA14_66250 - PA14_66060). Excepted *dnpA*, PA14_66150 and PA14_66190, these genes have a predicted or verified role in the assembly and regulation of the biosynthesis of LPS core molecules in *P. aeruginosa* (King *et al.*, 2009; Lam *et al.*, 2011).

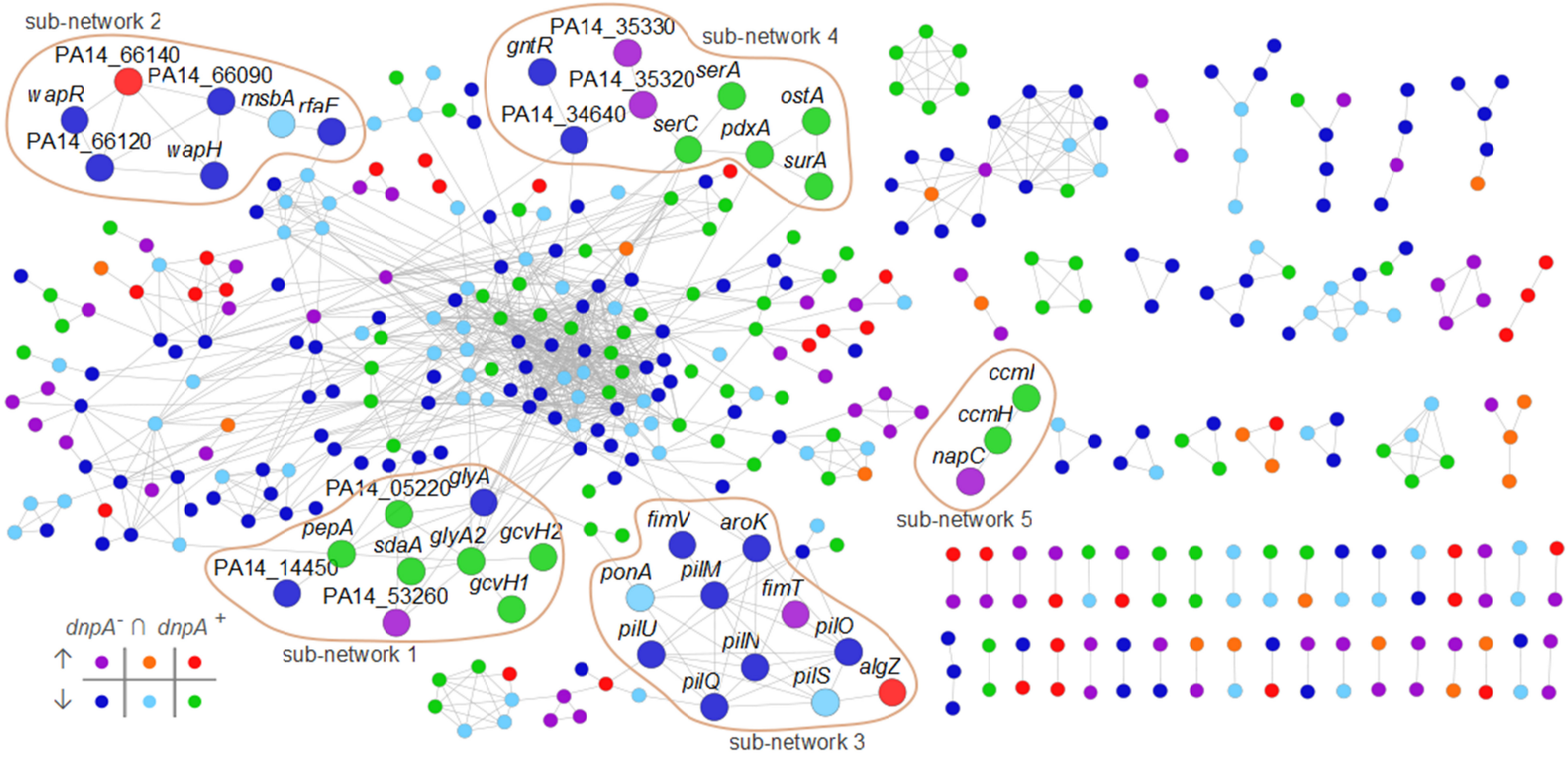


Figure 4.11: Differentially expressed genes mapped on the functional interaction network STRING. Dark blue and red coloured circles represent genes downregulated in *dnpA* mutant or *dnpA* overexpression strain, respectively. Upregulated genes in mutant and overexpression strain are represented by purple and green coloured circles. Light blue and orange circles define genes down or upregulated in both conditions respectively. The five selected sub-networks are indicated ($P < 0.05$) and gene names or PA14 gene numbers are shown.

Table 4.4: Genes differentially expressed in *dnpA* mutant (*dnpA*$^-$) or overexpressing strain (*dnpA*$^+$) for each selected sub-network

Gene number	Gene name	Product name and/or function	Fold change (log2)	
			dnpA$^-$ /WT	*dnpA*$^+$ /WT
Sub-network 1: Amino acid biosynthesis and metabolism				
PA14_05220/PA0399		Cystathionine beta-synthase		-0.78
PA14_14470/PA3831	*pepA*	Leucine aminopeptidase		-0.39
PA14_32985/PA2446	*gcvH2*	Glycine cleavage system protein H2		-0.57
PA14_33010/PA2444	*glyA2*	Serine hydroxymethyltransferase		-0.54
PA14_33030/PA2443	*sdaA*	L-serine dehydratase		-0.44
PA14_53260/PA0851		Hypothetical protein	0.29	
PA14_68860/PA5214	*gcvH1*	Glycine cleavage system protein H1		-0.29
Sub-network 2: Cell wall/LPS/Capsule				
PA14_66090/PA4998		Conserved hypothetical protein	-0.34	
PA14_66110/PA5000	*wapR*	alpha-1,3-rhamnosyltransferase WapR	-1.10	
PA14_66120/PA5001		Conserved hypothetical protein	-2.23	
PA14_66140/PA5002		Conserved hypothetical protein		4.21
PA14_66160/PA5004	*wapH*	Probable glycosyl transferase	-0.60	
PA14_66060/PA4996	*rfaE*	LPS biosynthesis protein RfaE	-0.41	
Sub-network 3: Motility and attachment; Two-component regulatory systems				
PA14_05190/PA0396	*pilU*	Twitching motility protein PilU	-0.55	
PA14_23830/PA3115	*fimV*	Motility protein FimV	-0.31	
PA14_66610/PA5039	*aroK*	Shikimate kinase	-0.37	
PA14_66620/PA5040	*pilQ*	Type 4 fimbrial biogenesis outer membrane protein PilQ precursor	-0.36	
PA14_66640/PA5042	*pilO*	Type 4 fimbrial biogenesis protein PilO	-0.34	
PA14_66650/PA5043	*pilN*	Type 4 fimbrial biogenesis protein PilN	-0.39	
PA14_66660/PA5044	*pilM*	Type 4 fimbrial biogenesis protein PilM	-0.54	

Table 4.4 - continued

Gene number	Gene name	Product name and/or function	Fold change (log2)	
			dnpA⁻ /WT	*dnpA*⁺ /WT
PA14_69480/PA5262	*fimS/algZ*	FimS, phosphorelay signal transduction system		0.33
Sub-network 4: Biosynthesis of cofactors; Amino acid biosynthesis; Adaptation and protection				
PA14_04110/PA0316	*serA*	D-3-phosphoglycerate dehydrogenase		-0.37
PA14_07740/PA0593	*pdxA*	Pyridoxal phosphate biosynthetic protein PdxA		-0.32
PA14_07760/PA0594	*surA*	Peptidyl-prolyl cis-trans isomerase SurA		-0.31
PA14_35330/PA2262		Probable 2-ketogluconate transporter	0.21	
PA14_35320/PA2263		Probable 2-hydroxyacid dehydrogenase	0.34	
PA14_23270/PA3167	*serC*	3-phosphoserine aminotransferase		-0.27
PA14_07770/PA0595	*ostA*	Organic solvent tolerance protein OstA precursor		-0.35
Sub-network 5: Energy metabolism				
PA14_45280/PA1483	*ccmI*	Cytochrome c-type biogenesis protein CcmI		-0.46
PA14_45290/PA1482	*ccmH*	Cytochrome c-type biogenesis protein CcmH		-0.55
PA14_49270/PA1172	*napC*	Cytochrome c-type protein NapC	0.46	

LPS is an important surface-associated molecule involved in a variety of bacterial processes of great clinical importance, such as virulence, immune response modulation, motility and biofilm formation (King *et al.*, 2009). *P. aeruginosa* has extensively been used as a model system to study this complex molecule and many of the biosynthesis and regulatory genes have been characterized. In addition, most of the transferase genes that are involved in the assembly of the core oligosaccharide are located in this gene cluster. The cluster is generally well conserved among *P. aeruginosa* strains, as is the structure of the LPS core

oligosaccharide (Kocincova & Lam, 2011). Surprisingly, no difference could be detected when comparing LPS profiles of *dnpA* mutant, *dnpA* overexpression strain and wild type. Interestingly, the deduced gene product of *E. coli* CFT073 *c2486* is also a member of the PIG-L superfamily. *c2486* is part of a gene cluster (*c2485-c2490*) that is required for hypercolonization in mice. It was postulated to encode an LPS modification system, but, as was the case in our study, no changes in LPS structure were observed for a mutant lacking *c2485-c2490* (Haugen *et al.*, 2007). It is possible that DnpA is responsible for minor modifications of the LPS core which do not affect the LPS profile. For instance, it has been described that a change in acetylation state does not affect LPS synthesis when evaluated by a LPS profile (Slauch *et al.*, 1995). On the other hand, *dnpA* could be involved in a process other than LPS core biosynthesis. PA66150-*dnpA* is conserved as one unit in all of the genomes analyzed but its genomic location relative to the cluster is not conserved. This is most obvious in *P. putida* W619 where parts of the *P. aeruginosa* cluster can be found in three discrete genomic locations separated by several hundreds of unrelated genes (Figure 4.4). This "uncoupling" of PA14_66150-*dnpA* from the validated core LPS genes lends indirect support for a role in a process other than LPS core biosynthesis. In fact, the PA14_66150 orthologue in *P. fluorescens* was attributed a role in the regulation of exopolysaccharide synthesis (Nian *et al.*, 2007). More recently, it was reported that PA14_66150 is involved in sensing cationic AMPs which leads to the induction of the LPS modification operon *arnBCADTEF*-PA3559 (Jochumsen *et al.*, 2011). This gene cluster is responsible for adding 4-amino-4-deoxy-L-arabinose to the phosphate group of lipid A, thereby increasing tolerance to cationic AMPs (Moskowitz *et al.*, 2004). It is possible that *dnpA*, together with PA14_66150, is involved in monitoring LPS biosynthesis/assembly or outer membrane physiology (Jochumsen *et al.*, 2011). Future research may reveal whether *dnpA* serves the same function in all *Pseudomonas* spp.

Sequence analysis revealed that DnpA belongs to the PIG-L or LmbE-like superfamily (Pfam PF02585). Based on a comparison between all biochemically characterized members of this family, we predict that DnpA acts as a de-*N*-acetylase on the *N*-acetylglucosamine part of a unknown substrate. All eukaryotic members studied so far play a role in the synthesis of glycosylphosphatidylinositol, a glycolipid that anchors proteins at the cell surface (Nakamura *et al.*, 1997; Sharma *et al.*, 1997; Smith *et al.*, 1997; Watanabe *et al.*, 1999). In bacteria, characterized PIG-L members are involved in different unrelated pathways such as chitine degradation (Tanaka *et al.*, 2004; Hou *et al.*, 2013), biosynthesis of low-molecular-weight

thiols (Newton *et al.*, 2000; Gaballa *et al.*, 2010) and synthesis of antibiotics (Ho *et al.*, 2006; Truman *et al.*, 2006; Truman *et al.*, 2007; Fan *et al.*, 2008). Based on mutational and crystal structure analysis of PIG-L members, a few models for the catalytic activity were proposed (Urbaniak *et al.*, 2005; Fadouloglou *et al.*, 2007; Zou *et al.*, 2008). They are all consistent with the mechanism of other zinc-dependent deacetylases (Hernick & Fierke, 2005). Briefly, zinc is pentacoordinated by two histidines (DnpA His-188 and His-341 in Figure 4.6), one aspartic acid (DnpA Asp-191 in Figure 4.6) and two water molecules. Upon substrate binding one water molecule is displaced. The second water molecule is activated by the catalytic base (DnpA Asp-190 in Figure 4.6) and a nucleophilic attack takes place after which a tetrahydral oxyanion intermediate is formed. Subsequent protonation of the leaving amino group completes the catalytic cycle, releasing the deacetylated substrate from the catalytic site. It has been suggested that the residue corresponding to His-338 interacts with the substrate and stabilizes the negative charge in the oxyanion intermediate. The Asp-340 residue is thought to be involved in this process by stabilising the positive charge on the His-338 residue (Zou *et al.*, 2008).

Overexpression of *dnpA* in wild-type background increases the persister fraction significantly. We investigated whether two mutant *dnpA* alleles are still capable of increasing the persister fraction upon overexpression. The corresponding mutations have been described to cause a significantly decreased enzymatic activity *in vitro* in other species (Urbaniak *et al.*, 2005; Deli *et al.*, 2010). Overexpression of the *dnpA*-D340N allele no longer increased the persister fraction in a wild-type background. This result indicates that the predicted de-*N*-acetylase activity is important in fluoroquinolone tolerance. Surprisingly, the D190N allele did not alter the persister fraction upon *dnpA* overexpression. This is not expected since D190 is postulated to function as the catalytic base. Mutation of this residue is supposed to completely abolish de-*N*-acetylase activity of the protein (Urbaniak *et al.*, 2005). However, it has been reported that mutation of this general catalytic base does not change the enzymatic activity of the *Entamoeba histolytica* PIG-L protein *in vitro* (Ashraf, 2013). This points to the possibility that the catalytic mechanism is not conserved among PIG-L members. To fully answer the question whether the predicted de-*N*-acetylase activity of DnpA is important in fluoroquinolone tolerance, data on *in vitro* biochemical activity are needed. However, we were not able to purify sufficient amount of DnpA to carry out such tests (as described in Appendix A).

A transcriptome analysis was carried out in order to gain more insight into the overall cellular function of DnpA and its role in fluoroquinolone tolerance. Differentially expressed genes were classified into functional categories. Enriched categories were identified and two of them, "Amino acid biosynthesis and metabolism" and "Energy metabolism", were shared between mutant and overexpression strain. Most of the genes belonging to the enriched classes were downregulated. In order to identify groups of genes which are consistently up/down (or down/up) regulated in the *dnpA* mutant and in the strain overexpressing *dnpA* respectively, a deregulated functional interaction network was constructed. The selected gene groups suggest processes that are consistently affected by both perturbations.

In general, genes involved in "Amino acid biosynthesis and metabolism" and "Energy metabolism" are downregulated in the *dnpA* overexpression strain suggesting a lower metabolic activity. This is consistent with earlier microarray analyses in which non-essential genes and genes involved in metabolism were downregulated in persisters (Keren *et al.*, 2004b; Shah *et al.*, 2006).

In the *dnpA* mutant, genes belonging to "Cell wall/LPS/Capsule" and "Motility and attachment" are downregulated. As is the case for *dnpA,* genes belonging to the first category are all situated in the LPS core oligosaccharide cluster. As mentioned earlier, no difference in LPS profile could, however, be detected. The second category contains genes involved in pilin biosynthesis. Previously, *pilH* and *algR* were identified as genes with a role in *P. aeruginosa* persistence. Insertion mutants of both genes displayed a higher persister fraction (De Groote *et al.*, 2009). PilH is a response regulator involved in regulation of pilus retraction (Bertrand *et al.*, 2010), while AlgR is part of a two-component transmitter-receiver regulatory system involved in regulation of alginate synthesis and twitching motility (Whitchurch *et al.*, 1996). Type IV pili are thin, flexible filaments with a broad range of functions including a role in motility, adherence and aggregation, protein secretion and biofilm formation and remodelling (Giltner *et al.*, 2012). Exactly how biosynthesis of type IV pili is related to fluoroquinolone tolerance remains an open question.

In summary, we have shown that *dnpA* is a *bona fide* persistence gene. Contrary to expectations, no obvious role in LPS biosynthesis could be demonstrated. DnpA is a putative de-*N*-acetylase, and this enzymatic activity is likely essential for its role in persistence. Other members of the PIG-L superfamily have previously been shown to be amenable to specific inhibition by small molecule compounds (de Macedo *et al.*, 2003; Metaferia *et al.*, 2007;

Gammon *et al.*, 2010). Together, our results offer the prospect of targeting DnpA in future anti-persister therapies to more efficiently combat recalcitrant *P. aeruginosa* infection.

Chapter 5

Membrane localization and topology of DnpA and its importance in fluoroquinolone tolerance in *Pseudomonas aeruginosa*[11]

5.1 Introduction

In the previous Chapter, we showed that *dnpA* is a bona fide persistence gene. However, currently, the exact function of this gene is still unknown. Genome-wide transcriptome analysis of strains lacking or overexpressing *dnpA* (see Chapter 4) points to a role in processes associated with the cell envelope (e.g. cell wall synthesis, motility and attachment). In a genome-wide computational screening for N-terminal signal peptides in *Pseudomonas aeruginosa*, DnpA was identified as one of the proteins predicted to use a transmembrane helix for membrane targeting (Lewenza *et al.*, 2005). The presence of DnpA within the membrane would provide further support for its role in a surface-associated process. Therefore, an important step in the functional study of DnpA is determining its subcellular localization as well as its topology.

In this Chapter, the predicted membrane localization and topology are investigated in detail. In addition, we evaluated whether correct membrane localization of DnpA is important for its role in fluoroquinolone tolerance. These results will help in understanding the role of *dnpA* in the complex persistence phenomenon in *P. aeruginosa*.

[11] Subcellular localization experiments with DnpAΔ60-PhoA, DnpAΔ125-PhoA and DnpAΔ178-PhoA described in this Chapter were performed by Dr. Emanuela Frangipani (University Roma Tre, Rome); The majority of the experiments described in this Chapter were carried out at the Molecular Microbiology Laboratory headed by Prof. Paolo Visca (University Roma Tre, Rome) in the framework of a research visit supported by a FEMS Research Fellowhip.

5.2 Experimental procedures

Strains and growth conditions. *P. aeruginosa* PA14 WT (Liberati *et al.*, 2006) was cultured in Luria-Bertani (LB) broth, for solidified medium 1.5 % agar was added. The following antibiotics were used: carbenicillin (300 µg mL^{-1}) and tetracycline (10 or 100 µg mL^{-1}). Plasmids used in this Chapter are listed in Table 5.1.

Table 5.1: Plasmids and encoded proteins used in this Chapter

Plasmid or construct	Description or encoded protein*	Reference
Plasmids		
pME6032	pVS1 derived shuttle vector; Tc^R	Heeb *et al.* (2000)
pPHO7	pTZ18R plasmid containing *phoA* lacking its N-terminal signal peptide; Ap^R	Gutierrez and Devedjian (1989)
pUCP18	*E. coli-Pseudomonas* shuttle vector derived from pUC18/19; Ap^R Cb^R	Schweizer (1991)
Constructs		
pME6032-*dnpA*-*phoA*	DnpA-PhoA	This chapter
pME6032-*dnpA*N178-*phoA*	DnpAN178-PhoA	This chapter
pME6032-*dnpA*N60-*phoA*	DnpAN60-PhoA	This chapter
pME6032-*dnpA*N17-*phoA*	DnpAN17-PhoA	This chapter
pME6032-*dnpA*N90-*phoA*	DnpAN90-PhoA	This chapter
pME6032-*dnpA*N125-*phoA*	DnpAN125-PhoA	This chapter
pME6032-*dnpA*N10-*phoA*	DnpAN10-PhoA	This chapter
pME6032-*dnpA*N12-*phoA*	DnpAN12-PhoA	This chapter
pME6032-*dnpA*N15-*phoA*	DnpAN15-PhoA	This chapter
pME6032-*dnpA*Δ178-*phoA*	DnpAΔ178-PhoA	This chapter
pME6032-*dnpA*Δ125-*phoA*	DnpAΔ125-PhoA	This chapter
pME6032-*dnpA*Δ60-*phoA*	DnpAΔ60-PhoA	This chapter

* Tc^R, tetracycline resistant; Ap^R, ampicillin resistant; Cb^R, carbenicillin resistant

***In silico* analysis.** The hydrophobic character of DnpA was analysed using the Kyte-Doolittle method (Kyte & Doolittle, 1982). A window size of 19 amino acids was applied in order to

identify putative hydrophobic membrane-spanning domains (http://web.expasy.org/protscale). Transmembrane regions were predicted using TmPred (Hofmann & Stoffel, 1993), TMHMM (Krogh *et al.*, 2001) and DAS (Cserzo *et al.*, 1997) (available on http://web.expasy.org/tools/). The presence of signal peptide cleavage sites was examined by using Signal4P (Petersen *et al.*, 2011), default settings were used for D-cutoff values and minimal predicted signal peptide length.

Construction of DnpA-PhoA translational fusions. Several N- and C-terminal fragments of DnpA were fused in frame with PhoA. For each fusion, the desired *dnpA* fragment was amplified using primers as listed in Table 5.2 with *P. aeruginosa* PA14 genomic DNA as a template. In each primer an appropriate restriction site was included which allowed directional incorporation of the resulting PCR product into the compatible restriction site of pME6032 using EcoRI and BglII. DnpAN10, DnpAN12 and DnpAN15 fragments were constructed using the oligonucleotide adaptor technique (Invitrogen). Subsequently, the *phoA* reporter sequence from pPHO7 was cloned downstream of the N- or C-terminal fragment between the XhoI site of pME6032 and the HindIII sites incorporated in the sense primer used to construct each *dnpA* fragment. The resulting fusions were confirmed by sequencing. Plasmids were introduced into *P. aeruginosa* PA14 WT by transformation. In addition, all resulting strains were transformed with the plasmid pUCP18 in order to obtain constitutive ß-lactamase production.

Table 5.2: Primers used in this Chapter

Primer name	Sequence *	Description	Restriction site
SPI6628	CACC**GAATTC**AGCATGAG CGCACGCAAG	Antisense primer for amplification of N-terminal fragments	EcoRI
SPI10088	ACTC**AGATCTAAGCTT**A GCGCCTCCCCATCCAG	Sense primer for amplification of full length *dnpA*	BglII and HindIII
SPI10089	ACTC**AGATCTAAGCTT**A GACGGTAATCGGGATGGC	Sense primer for amplification of *dnpA*N178	BglII and HindIII

Table 5.2 - continued

Primer name	Sequence *	Description	Restriction site
SPI10090	ACTC**AGATCTAAGCTT**A GATAGAACAGGTGATCGG CG	Sense primer for amplification of *dnpA*N60	BglII and HindIII
SPI10092	ACTC**AGATCTAAGCTT**A GCCGCTTGTTGC	Sense primer for amplification of *dnpA*N17	BglII and HindIII
SPI10152	ACTC**AGATCTAAGCTT**A GGCCGTCGACTCGC	Sense primer for amplification of *dnpA*N90	BglII and HindIII
SPI10153	ACTC**AGATCTAAGCTT**A GGGCCTCCGCGC	Sense primer for amplification of *dnpA*N125	BglII and HindIII
SPI10365	**AATTC**AGCATGAGCGCAC GCAAGCAGCAGTTGCTCA AGCT**AAGCTTA**	Antisense oligonucleotide *dnpA*N10	EcoRI, HindIII and BglIII
SPI10366	**GATCTAAGCTT**AGCTTGA GCAACTGCTGCTTGCGTG CGCTCATGCT**G**	Sense oligonucleotide *dnpA*N10	BglII, HindIII and EcoRI
SPI10367	**AATTC**AGCATGAGCGCAC GCAAGCAGCAGTTGCTCA AGCGTCATCT**AAGCTTA**	Antisense oligonucleotide *dnpA*N12	EcoRI, HindIII and BglIII
SPI10368	**GATCTAAGCTT**AGATGA CGCTTGAGCAACTGCTGC TTGCGTGCGCTCATGCT**G**	Sense oligonucleotide *dnpA*N12	BglII, HindIII and EcoRI
SPI10369	**AATTC**AGCATGAGCGCAC GCAAGCAGCAGTTGCTCA AGCGTCATCGACGCAAC CT**AAGCTTA**	Antisense oligonucleotide *dnpA*N15	EcoRI, HindIII and BglIII
SPI10370	**GATCTAAGCTT**AGGTTGC GTCGATGACGCTTGAGCA ACTGCTGCTTGCGTGCGC TCATGCT**G**	Sense oligonucleotide *dnpA*N15	BglII, HindIII and EcoRI
SPI10174	CACC**GAATTC**ATGTCGCC GGGCGAGGACTACCGCTA CCGTTTC	Antisense primer for amplification of *dnpA*Δ60	EcoRI

Table 5.2 - continued

Primer name	Sequence *	Description	Restriction site
SPI10175	CACC**GAATTC**ATGGATGC CCAGGCTTTCGAACGTGG	Antisense primer for amplification of *dnpA*Δ125	EcoRI
SPI10176	CACC**GAATTC**ATGGAGCG GCGGGTGATGGTCATCG	Antisense primer for amplification of *dnpA*Δ178	EcoRI

*(Partial) restriction sites are indicated in bold

Isolation of membrane fractions. This procedure was carried out as described in Chapter 4. The presence of DnpA-PhoA and the inner membrane marker XcpY (Filloux *et al.*, 1990) in each fraction was analyzed by western hybridization using respectively anti-PhoA (Sigma) and anti-XcpY (kind gift from Dr. Romé Voulhoux, Aix-Marseille Université) as primary antibodies.

Subcellular fractionation. Overnight cultures were diluted 100-fold into LB medium and incubated at 37 °C while shaking at 200 rpm. At an OD_{595} of 0.5, cultures were induced with 1 mM isopropyl ß-D -1-thiogalactopyranoside (IPTG) for one hour at 37 °C after which the cells were harvested by centrifugation (20 minutes at 5000 rpm). Subcellular fractions were isolated as described by Imperi *et al.* (2008) with minor adaptations. Briefly, cells were spheroplasted by the lysozyme/sucrose method (Robles-Price *et al.*, 2004) to release periplasmic proteins. Next, spheroplasts were disrupted by sonication and cell debris were removed by low-speed centrifugation (10 min at 3000 ***g***). Separation of the cytosolic and membrane fractions was carried out by ultracentrifugation at 100,000 ***g*** for one hour at 4 °C. The purity of subcellular fractions was verified by measuring relative isocitrate dehydrogenase, lactate dehydrogenase and ß-lactamase activities. Equal volumes of each fraction were separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) analysis after which the presence of DnpA-PhoA in each fraction was verified by immunoblot analysis with anti-PhoA (Sigma) as a primary antibody.

Enzymatic assays. Isocitrate dehydrogenase activity was measured as described previously (Goldberg & Ellis, 1983) with minor modifications. Essentially, the enzymatic activity was measured by monitoring the reduction of $NADP^+$ to NADPH during conversion of isocitrate to 2-oxoglutarate. The increase in NADPH production was monitored by measuring the

absorbance at 339 nm over time. Reactions were carried out at room temperature in a 0.065 M triethanolamine buffer (pH = 7.3) containing 1.66 mM $MnCl_2$. ß-lactamase activity was determined essentially as described by O'Callaghan *et al.* (1972) with minor adjustments. Briefly, enzymatic activity was measured by monitoring cleavage of nitrocefin, a chromogenic cephalosporin substrate, by evaluating the increase in absorbance at 486 nm over time. The enzymatic assay was carried out at room temperature in a 50 mM Na-phosphate buffer (pH = 7). Lactate dehydrogenase activity was assessed using a modification of the Vassault (1983) protocol. This enzyme converts pyruvate into lactate resulting in the oxidation of NADH to NAD^+. This decrease of NADH was determined by measuring the absorbance at 339 nm over time at room temperature. The enzymatic reaction was carried out in a 80 mM Tris/200 mM NaCl (pH = 7.2) solution.

Membrane association assay. The biochemical nature of the DnpA membrane association was evaluated by treating total membrane fractions of cells overexpressing the DnpA-PhoA fusion protein with different chemical agents as described by Imperi *et al.* (2008) with minor modifications. Briefly, membrane fractions were treated for 30 minutes at 4 °C with 1 % SDS, 2 % Sarcosyl, 1 M NaCl, 1.5 M and 5 M urea and 0.01 M NaOH. Next, treated samples were centrifuged at 55,000 ***g*** for two hours at 4 °C. Equal volumes of pellet and supernatant were separated by SDS-PAGE and analyzed for the presence of DnpA-PhoA by immunoblot analysis using a primary antibody targeting PhoA (Sigma).

PhoA activity. Overnight cultures were diluted 100-fold into LB medium and incubated at 37 °C while shaking at 200 rpm. At an OD_{595} of 0.2, IPTG was added to the bacterial cultures to a final concentration of 1 mM. After two hours of induction, 5 µl of each culture was spotted onto LB agar plates containing 40 µg mL^{-1} 5-bromo-4-chloro-3-indolyl phosphate (BCIP), 1 mM IPTG and the appropriate antibiotics. Additionally, KH_2PO_4 was added to a final concentration of 10 mM to inhibit the endogenous phosphatase activity. Expression of the respective fusion proteins was verified by western hybridization using a primary antibody targeting PhoA (Sigma).

Persistence assay. Determination of the persister fraction was carried out as described in Chapter 4.

Statistical analysis was performed as described in Chapter 4.

5.3 Results

5.3.1 *In silico* analysis of DnpA

Analysis of a hydrophobicity plot of the DnpA amino acid sequence, calculated by the Kyte-Doolittle algorithm, revealed a highly hydrophobic peak encompassing amino acids 10-50 (Figure 5.1A). This hydrophobic region corresponds to a putative transmembrane domain predicted by TmPred, TMHMM (Figure 5.1B and C) and DAS. These algorithms predict one putative transmembrane helix and, additionally, TMHMM and TmPred strongly suggest an N_{in}/C_{out} orientation of DnpA meaning that the majority of the protein is predicted to reside in the periplasmic space. When analyzing the DnpA amino acid sequence by Signal4P, no putative signal peptide cleavage site was detected.

5.3.2 Membrane localization of DnpA

To verify the localization of DnpA in the cytoplasmic membrane, the full-length *dnpA* gene was coupled in-frame with *phoA*, which encodes a reporter enzyme. Inner and outer membrane fractions of *P. aeruginosa* cells overexpressing this fusion protein were isolated. The resulting fractions were separated on SDS-PAGE after which the presence of the fusion protein was evaluated by western hybridization using anti-PhoA antibodies as a primary antibody (Figure 5.2A). DnpA-PhoA was present in the whole cell lysate and inner membrane fraction, confirming the predicted localization in the cytoplasmic membrane. As expected, the same result was obtained when the presence of the known inner membrane protein XcpY (Filloux *et al.*, 1990) was evaluated in these fractions.

5.3.3 Orientation of DnpA in the cytoplasmic membrane

In order to determine the orientation of DnpA in the cytoplasmic membrane, the enzymatic activity of the C-terminally coupled fusion protein DnpA-PhoA was examined. PhoA is an enzyme that only displays activity when located in the periplasm. The PhoA fragment used here lacks its signal sequence. It therefore folds and thus becomes enzymatically active only when the fusion protein DnpA-PhoA is exposed at the periplasmic side of the membrane (Manoil *et al.*, 1990). *P. aeruginosa* cells overexpressing the fusion protein were plated on

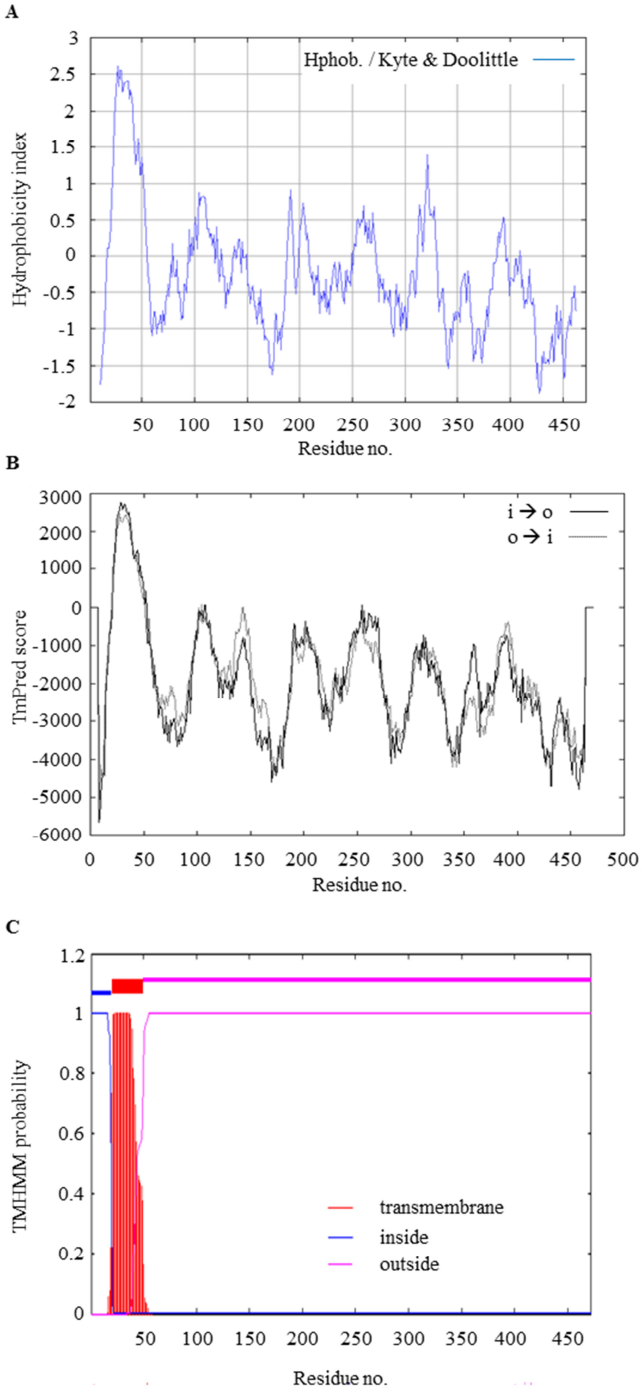


Figure 5.1: Hydrophobicity plot and transmembrane domain predictions for DnpA of *Pseudomonas aeruginosa*. A Hydrophobicity plot generated by the Kyte and Doolittle algorithm which gives a score for the hydrophobic character of each amino acid of DnpA. **B.** Prediction of transmembrane domains by TmPred with a TmPred score assigned for each DnpA residue. Only scores > 500 are considered significant and indicate that the residue likely belongs to a transmembrane helix. **C.** Predictions of transmembrane domains by the TMHMM algorithm in which a probability score is given for the transmembrane (red), inside (blue) and outside (pink) location for each amino acid residue of DnpA. At the top of the plot the best prediction is shown. GenBank protein accession number NP_253689.

solid growth medium containing the PhoA substrate BCIP, which is converted into a blue precipitate upon phosphatase activity. The DnpA-PhoA fusion protein does not display enzymatic activity, indicating that the C-terminal part of DnpA is located at the cytoplasmic side of the inner membrane (Figure 5.2B).

5.3.4 Membrane association of DnpA

To further characterize the biochemical association of DnpA with the cytoplasmic membrane, total membrane fractions of *P. aeruginosa* cells overexpressing DnpA-PhoA were treated with different chemical agents (Figure 5.3). DnpA-PhoA was released from the membrane fraction upon treatment with the detergents SDS (1 %) and Sarcosyl (2 %). The latter specifically disrupts the inner membrane, hereby independently confirming the inner membrane localization of DnpA. Treatment of membrane fractions with NaCl (1 M), which specifically disrupts protein–protein and protein–phospholipid interactions, did not cause solubilization of DnpA-PhoA. In addition, neither did treatment with denaturant (1.5 M and 5 M urea) or alkali (0.01 M NaOH), which specifically cause solubilization of peripheral membrane proteins. Taken together, these results indicate that DnpA is an integral membrane protein.

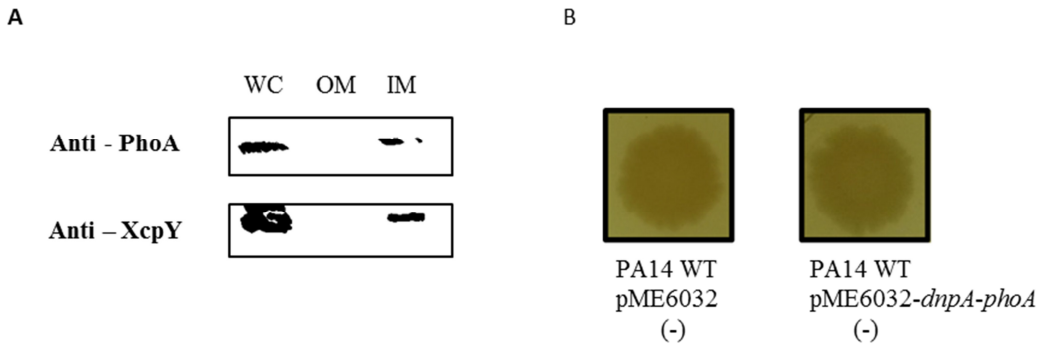


Figure 5.2: Membrane localization and orientation of DnpA. A. Western hybridization analysis of whole cell lysates (WC) and inner (IM) and outer (OM) membrane fractions of *P. aeruginosa* cells overexpressing the DnpA-PhoA fusion protein (theoretical molecular weight of 102.55 kDa). Equal volumes of each fraction were analyzed. Analysis was carried out with an anti-PhoA or anti-XcpY primary antibody as indicated. **B.** Phosphatase activity of IPTG-induced cells containing the plasmid pME6032 or pME6032-*dnpA-phoA*. The absence of phosphatase activity is indicated by (–).

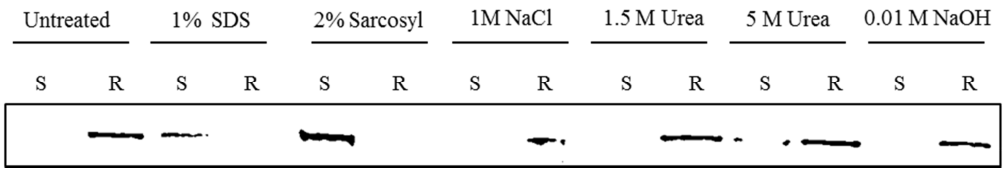


Figure 5.3: Membrane association of DnpA. Total membrane fraction of cells overexpressing DnpA-PhoA from the plasmid pME6032 were treated with the different detergents indicated. The resulting pellet was resuspended in an equal volume of buffer. Pellet and supernatant were subjected to western hybridization analysis using an anti-PhoA antibody. Theoretical molecular weight of DnpA-PhoA is 102.55 kDa.

5.3.5 Topology of DnpA

5.3.5.1 Confirmation of the predicted model

To confirm the presence of the predicted transmembrane domain, different N-terminal fragments of DnpA were fused in-frame with the reporter enzyme PhoA, which lacks its native signal peptide. To prevent disruption of helical structures which might be involved in transmembrane segments, the secondary structure of DnpA as predicted by JPred (Cole *et al.*, 2008), was consulted. Fusion points were chosen before and after the predicted transmembrane domain and just before the start of the predicted PIG-L domain (Figure 5.4). Expression after induction from pME6032 was verified by western hybridization analysis using an anti-PhoA antibody. DnpAN15-PhoA was not expressed at detectable levels and therefore excluded from further testing.

The subcellular localization of each fusion protein was experimentally determined (Figure 5.5A). DnpAN60-PhoA, containing the first 60 N-terminal amino acid residues of DnpA fused to PhoA, is predominantly located in the membrane fraction, which confirms the presence of the predicted transmembrane helix. As expected, DnpAN178-PhoA, as well as DnpA-PhoA, is also dominantly present in the membrane fraction. To verify the cytoplasmic localization of the DnpA N-terminus, the following additional translational fusions were constructed: *dnpA*N10-*phoA*, *dnpA*N12-*phoA* and *dnpA*N17-*phoA*. When assessing expression of these fusion proteins, none of them could be detected (data not shown). To independently provide support for the presence of the predicted transmembrane helix, inner and outer membrane fractions of cells overexpressing DnpAN60-PhoA were isolated. The fusion protein was exclusively detected in the inner membrane fraction of the cell (Figure

5.5B) as was the inner membrane marker XcpY. This indicates the presence of a transmembrane domain in the 60 N-terminal amino acid residues of the protein.

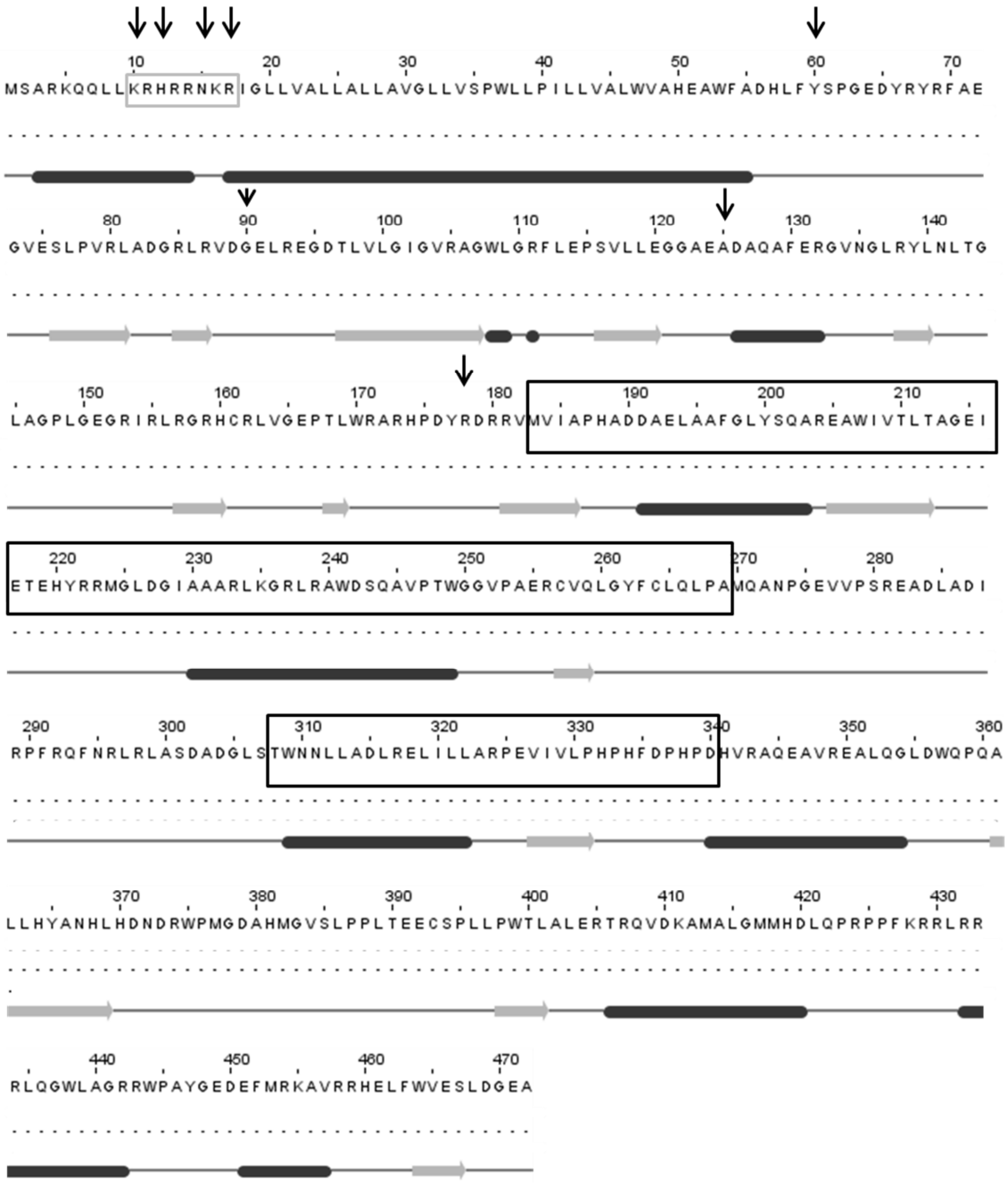


Figure 5.4: Secondary structure prediction of DnpA. Light grey horizontal arrows represent predicted strands, helices are visualized by dark grey bars. Vertical arrows indicate fusion points used for the reporter enzyme encoded by *phoA*. Residues belonging to the predicted PIG-L domain are enclosed in the dark grey rectangle, a stretch of positively charged amino acid residues preceding the first predicted transmembrane helix is boxed in the light grey rectangle. The prediction was visualized using Jalview (Waterhouse *et al.*, 2009).

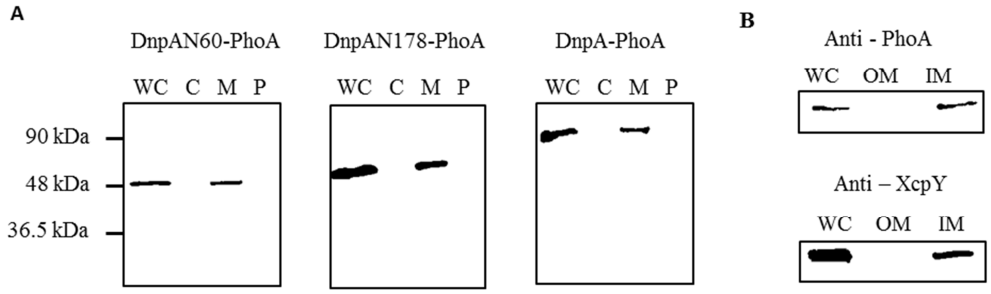


Figure 5.5: Subcellular localization of DnpA-PhoA fusion proteins. A. Immunoblot analysis of the different subcellular fractions of cells overexpressing the indicated DnpA-PhoA fusions proteins. Equal volumes of whole cell lysate (WC), cytoplasmic fraction (C), membrane fraction (M) and periplasmic fraction (P) were loaded onto protein gels. Immunoblot analysis was carried out with an anti-PhoA antibody. **B.** Western hybridization analysis of whole cell lysates (WC) and inner (IM) and outer (OM) membrane fractions of *P. aeruginosa* cells overexpressing the DnpAN60-PhoA fusion protein. Equal volumes of each fraction were analyzed. Immunoblot analysis was carried out with an anti-PhoA and anti-XcpY primary antibody. Theoretical molecular weight of 55.92 kDa, 68.97 kDa and 102.55 kDa for DnpAN60, DnpAN178 and DnpA-PhoA respectively.

Next, the phosphatase activity of each fusion protein was determined by plating the cells on BCIP-containing plates (Figure 5.6). *P. aeruginosa* cells overexpressing DnpAN60-PhoA are clearly able to convert BCIP into a blue precipitate, meaning that the C-terminal part of the DnpAN60 is located at the periplasmic side of the membrane. DnpAN178-PhoA overexpressing cells did not show enzymatic activity, indicative of a cytoplasmic localization of the DnpAN178 C-terminal part. This observation suggests the presence of an additional transmembrane segment between amino acid residues 60 and 178.

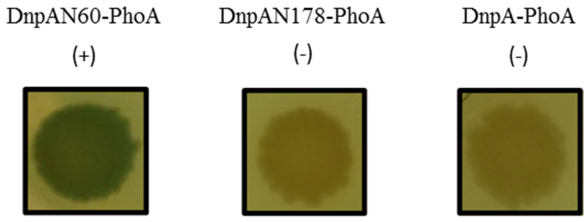


Figure 5.6: Enzymatic activity of DnpA-PhoA fusion proteins. Enzymatic activity of cells overexpressing the indicated fusion proteins after plating on BCIP containing agar plates. The presence or absence of phosphatase activity is indicated by (+) or (–) respectively.

5.3.5.2 Confirmation of second transmembrane domain

When studying the predictions made by TmPred and DAS, an additional transmembrane helix encompassing amino acids 98-119, although indicated as statistically insignificant, was detected. This amino acid region contains hydrophobic residues, further lending support for

an additional transmembrane domain. However, analysis of the predicted secondary structure does not reveal the presence of a helix. To examine the presence of a transmembrane domain, two additional translational fusions, *dnpA*N90-*phoA* and *dnpA*N125-*phoA*, were constructed. As expected, both fusion proteins were predominantly situated in the membrane fraction of *P. aeruginosa* cells. However, phosphatase activity tests were negative (Figure 5.7A), meaning that the C-terminus of DnpAN90 and DnpAN125 resides at the cytoplasmic side of the membrane. As cells overexpressing DnpAN60-PhoA showed phosphatase activity, our results indicate the presence of a transmembrane segment located between amino acid 60 and 90. To independently confirm the presence of this second membrane-crossing domain, the subcellular localization of DnpA lacking amino acids 1 to 60, was determined (Figure 5.7B). The result shows that this protein, DnpAΔ60-PhoA, predominantly resides in the membrane fraction, consistent with the presence of a second transmembrane segment downstream of amino acid residue 60. However, we also observed the presence of part of the fusion protein in the periplasmic fraction. Possibly, this second transmembrane domain requires the first one for correct insertion into the membrane.

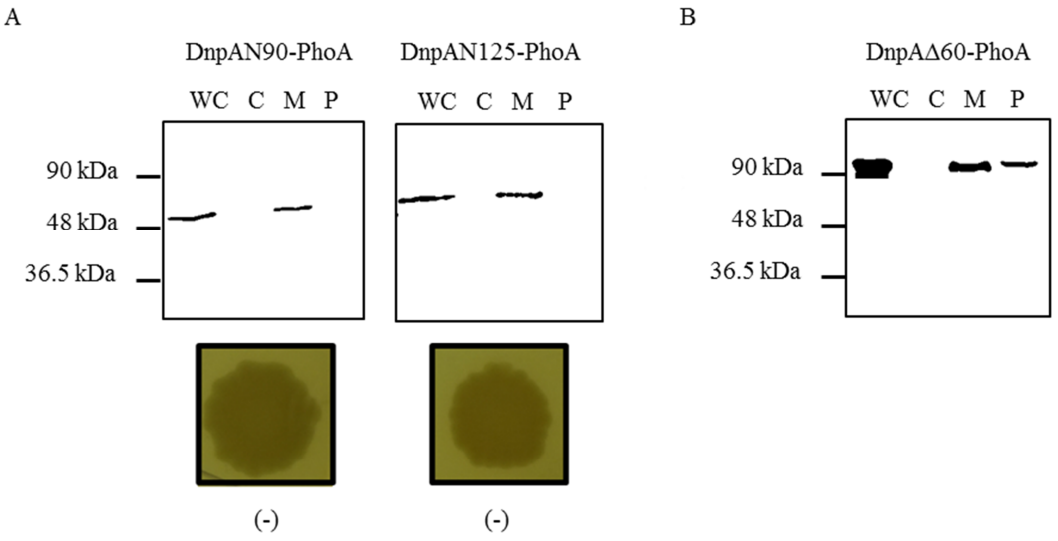


Figure 5.7: Evaluation of DnpAN90-PhoA, DnpAN125-PhoA and DnpAΔ60-PhoA. A. Subcellular localization and phosphatase activity of DnpAN90-PhoA and DnpAN125-PhoA (theoretical molecular weight of 60.46 kDa and 64.13 kDa respectively). Equal volumes of whole cell lysate (WC), cytoplasmic fraction (C), membrane fraction (M) and periplasmic fraction (P) were loaded onto protein gels. Immunoblot analysis was carried out with an anti-PhoA antibody. Enzymatic activity of cells overexpressing the indicated fusion proteins after plating on BCIP containing agar plates. The absence of phosphatase activity is indicated by (–). **B.** Subcellular localization of cells overexpressing DnpAΔ60-PhoA (theoretical molecular weight of 96.95 kDa). Membrane fraction is 1:2 diluted compared to the other samples. Immunoblot analysis was carried out with an anti-PhoA antibody.

5.3.6 Importance of membrane localization of DnpA in fluoroquinolone tolerance

Overexpression of *dnpA* in a wild-type background increases the persister fraction significantly (see Section 4.3.1). To evaluate if membrane localization of DnpA is important for its role in fluoroquinolone tolerance, we evaluated the persister fraction upon overexpression of *dnpA* alleles lacking the first (*dnpA*Δ60-*phoA*) or both transmembrane domains (*dnpA*Δ125-*phoA* and *dnpA*Δ178-*phoA*). Only overexpression of DnpA-PhoA increased the persister fraction significantly compared to the wild type ($P < 0.05$) (Figure 5.8A). Next, the subcellular localization of DnpAΔ125-PhoA and DnpAΔ178-PhoA was examined. Unexpectedly, both were dominantly present in the membrane fraction (Figure 5.8B).

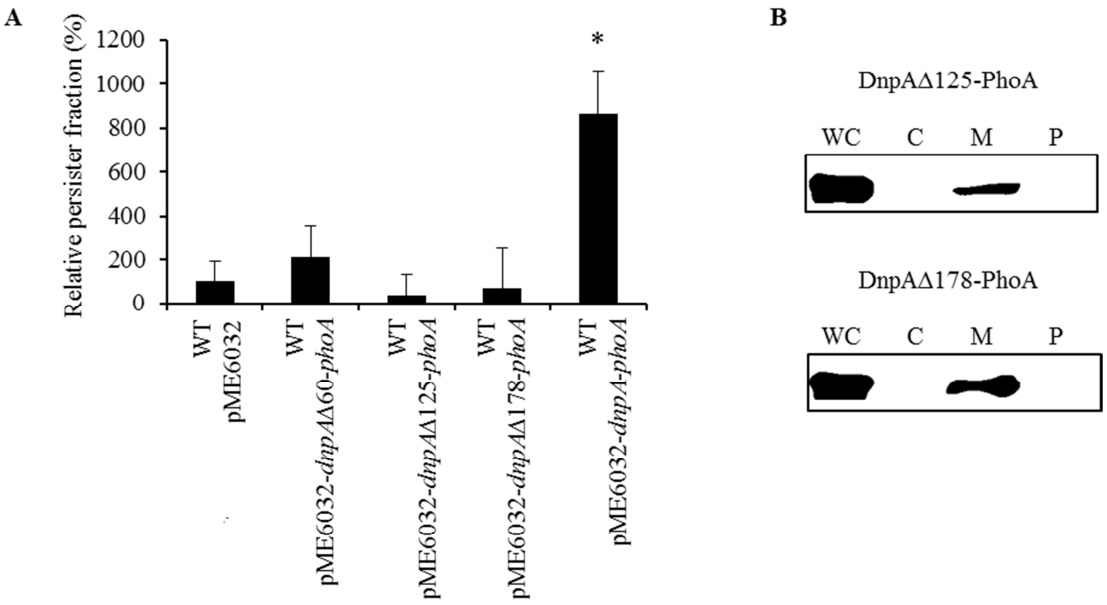


Figure 5.8: Relative persister fractions of cells overexpressing DnpA-PhoA fusion proteins lacking one or both transmembrane domains and subcellular localization of DnpAΔ125-PhoA and DnpAΔ178-PhoA. A. Stationary phase cells of wild type (WT) containing plasmid pME6032 (WT pME6032) and WT overexpressing *dnpA*Δ125-*phoA, dnpA*Δ178-*phoA* and *dnpA-phoA* respectively from the plasmid pME6032 (WT pME6032-*dnpA*Δx-*phoA*) were treated in parallel with ofloxacin and sterile water for 5 hours. Subsequently, the cells were diluted and plated out after which the number of surviving cells was determined. Data points correspond to the mean relative persister fraction. Each experiment was repeated independently at least three times. Error bars represent standard error of the mean. A persister fraction of 100 % corresponds to 6.81×10^{-5}. *$P < 0.05$. **B.** Subcellular localization of DnpAΔ125-PhoA and DnpAΔ178-PhoA (theoretical molecular weight of 89.92 kDa and 83.90 kDa respectively). An aliquot of whole cell lysate (WC), cytoplasmic fraction (C), membrane fraction (M) and periplasmic fraction (P) were loaded onto protein gels. The membrane fraction was diluted two times compared to the other samples. Immunoblot analysis was carried out with an anti-PhoA antibody.

5.4 Discussion

dnpA is involved in fluoroquinolone tolerance in *P. aeruginosa*, hereby contributing to the recalcitrant nature of infections caused by this pathogen. The role and cellular function of *dnpA* are currently unknown. Transcriptome analysis points to a possible role for DnpA in cell surface-associated processes. This is supported by its conserved genomic localization in the lipopolysaccharide (LPS) core oligosaccharide cluster, which is involved in synthesis of the extracellular factor LPS. In a genome-wide computational screening for N-terminal signal peptides in *P. aeruginosa*, DnpA was picked up as one of the proteins predicted to use a transmembrane helix for membrane targeting (Lewenza *et al.*, 2005). Presence of DnpA within the membrane would further lend support for a role of *dnpA* in cell-surface associated processes. However, to date, no experimental evidence is available in support of this prediction. In this Chapter, the subcellular localization of DnpA was investigated in detail as well as its relation to persistence.

All algorithms used predict the presence of a single transmembrane helix which anchors DnpA to the inner membrane, leaving the major part of DnpA at the periplasmic side of the lipid bilayer. Our test results confirm the presence of DnpA in the inner membrane but indicate an opposite orientation in which the C-terminal part of DnpA, which contains the PIG-L domain, is located in the cytoplasmic compartment. Errors regarding the orientation of the protein are amongst the most commonly ones made by the TMHMM algorithm (Krogh *et al.*, 2001), the best rated program for prediction of transmembrane helices (Moller *et al.*, 2001).

To confirm the presence and orientation of the predicted transmembrane helix, the reporter tag *phoA* was translationally fused to different N-terminal truncated variants of *dnpA*. *P. aeruginosa* cells overexpressing DnpAN60-PhoA clearly showed phosphatase activity on the substrate BCIP, confirming the periplasmic localization of the DnpAN60 C-terminal part. The cytoplasmic localization of the N-terminal part could not be confirmed since the fusion proteins DnpAN10-PhoA, DnpAN12-PhoA, DnpAN15-PhoA or DnpAN17-PhoA were not expressed. However, a stretch of positively charged amino acids residues (between residue 9 and 18)(Figure 5.4) precedes the transmembrane helix, which, according to the positive-inside rule (von Heijne, 1992), provides evidence for the cytoplasmic localization of the DnpA N-

terminal part. In addition, our results point to the presence of a second transmembrane domain downstream of amino acid residue 60, which we mapped between residues 60 and 90. This protein region neither contains a high number of hydrophobic amino acid residues, nor is it predicted to form a helix. However, increasing evidence is provided that marginally hydrophobic or helical segments can become incorporated in the membrane as well (von Heijne, 2011; Bogdanov *et al.*, 2014). Incorporation of these segments depends on the flanking sequences or, in some cases, on interaction with other transmembrane segments of the protein. As DnpAΔ60-PhoA lacks the first transmembrane domain and consequently its N-terminal flanking sequence, this may influence correct transfer or insertion of the second transmembrane segment into the membrane. This may account for the observed presence of the truncated form in the periplasm. The hydrophobic amino acid stretch between residues 90 and 125 possibly represents a region buried within the protein or associates with the cytoplasmic membrane without crossing it.

Based on *in silico* predictions and our experimental results, we suggest that DnpA possesses two transmembrane domains situated between amino acid residues 18-50 and 60-90 with an N_{in}/C_{out} and N_{out}/C_{in} orientation respectively. The majority of DnpA, including the PIG-L domain, is situated at the cytoplasmic side of the inner membrane (Figure 5.9).

Overexpression of the full length *dnpA* allele increases the persister fraction in a wild-type background, while overexpression of *dnpA* alleles lacking both transmembrane domains does not increase the persister fraction. This result suggests that correct membrane localization of DnpA is necessary for its effect on persistence. Surprisingly, unlike predicted, subcellular localization analysis of fusion proteins lacking both transmembrane domains revealed that these fragments were still located in the membrane fraction. Possibly, additional hydrophobic domains anchor DnpA peripherally to the cytoplasmic membrane. Based on Figure 5.1A, several candidate hydrophobic domains are present in DnpA which may fulfill this role. Additional experiments are needed to confirm this interaction of DnpAΔ125-PhoA and DnpAΔ178-PhoA with the membrane. For instance, treatment with alkali and denaturant should solubilize peripheral membrane proteins (Rosenberg, 2005; Imperi *et al.*, 2008). The presence of such additional hydrophobic domains would provide an explanation for the problematic purification of DnpA isoforms lacking the two transmembrane segments after overexpression in *Escherichia c*oli (as described in Appendix A). Alternatively, DnpA may be

bound by another membrane protein, hereby anchoring it to the membrane even in the absence of its two transmembrane segments. Taken together, correct transmembrane insertion of DnpA, but not membrane association *per se*, is needed to exert its effect on quinolone-tolerance in *P. aeruginosa*.

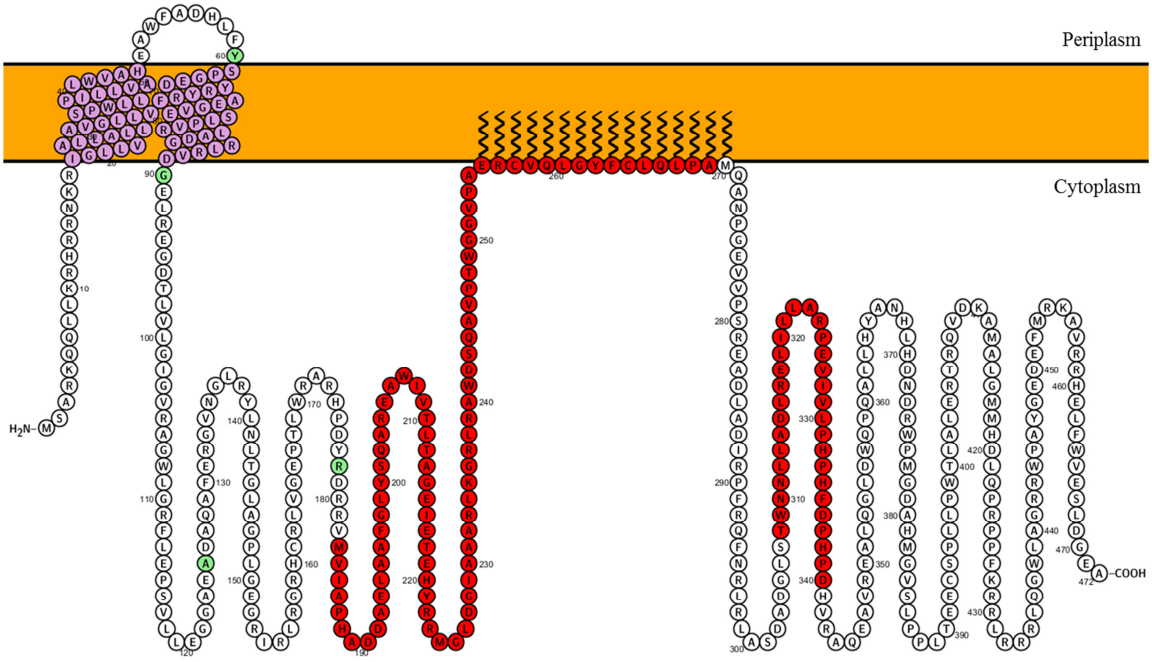


Figure 5.9: Topological map of *P. aeruginosa* DnpA based on *in silico* analysis, subcellular localization experiments and *phoA* fusion analysis. Purple regions represent transmembrane domains, green amino acids indicate fusion points for *phoA* and red amino acids belong to the PIG-L domain. The cytoplasmic membrane is shown in orange. This topological map was generated using Protter (Omasits *et al.*, 2013). Transmembrane domains were manually entered based on predictions made by TmPred and DAS and our experimental results. GenBank protein accession number NP_253689. To reflect the possible peripheral association of the DnpA C-terminus with the inner membrane, one of the candidate hydrophobic domains (amino acid residues 255-270), is shown to interact with the membrane.

5.5 Concluding remarks

We showed that DnpA resides in the inner membrane of *P. aeruginosa* by means of two transmembrane domains with the majority of DnpA including the catalytic domain located in the cytoplasmic compartment. Future experiments, in which the membrane association of *dnpA* alleles lacking the two transmembrane domains is investigated, will shed more light on

additional DnpA-membrane or DnpA-protein interactions. Furthermore, the observation that the catalytic domain of DnpA is located at the cytoplasmic face of the membrane may in the future be helpful to understand the cellular function of DnpA.

Chapter 6

Identification and characterization of anti-persister molecules

6.1 Introduction

Screening of knockout libraries, both in *Pseudomonas aeruginosa* (De Groote *et al.*, 2009) and *Escherichia coli* (Hu & Coates, 2005; Hansen *et al.*, 2008), did not result in the identification of mutants completely lacking persister cells, indicating the involvement of multiple mechanisms in persister formation. Furthermore, different bacteria use different mechanisms to form persister cells (See Chapter 2). For example, a major mechanism of persister formation in *E. coli*, a model system for persistence, involves toxin-antitoxin (TA) modules (Maisonneuve *et al.*, 2011; Maisonneuve *et al.*, 2013). However, the involvement of TA modules in *P. aeruginosa* has not been reported. Until now, the mechanism of persister formation in *P. aeruginosa* is largely unknown (as reviewed by Fauvart *et al.* (2011) and Mulcahy *et al.* (2013)) impeding the development of a target-based strategy against these antibiotic-insensitive cells. Additionally, persister cells are generally considered to be non-dividing, metabolically quiescent cells in which inactivity of the antibacterial targets impairs the bactericidal effect of currently known antibiotics (Bigger, 1944; Balaban *et al.*, 2004; Keren *et al.*, 2004b; Shah *et al.*, 2006; Lewis, 2007; Wood *et al.*, 2013). Consequently, no targets are available for developing a rational anti-persister strategy.

In this Chapter, using an untargeted approach, the identification and further characterization of small molecules with promising anti-persister properties is described. To identify anti-persister molecules, we conducted a medium-throughput screening in search of small molecules that, in combination with a clinically used antibiotic, specifically kill *P. aeruginosa* persister cells. These compounds can act as a starting point for the development of an anti-persister therapy. Additionally, by unraveling their mode of action, more insight is generated

into the mechanisms underlying persistence in *P. aeruginosa*. This may reveal molecular targets that will allow the future development of target-based anti-persister strategies. Since new anti-pseudomonal compounds are limited (Section 1.4.1.4), a parallel screening was carried out for growth inhibitory compounds. Identification and initial characterization of a promising compound is described in Addendum I.

6.2 Experimental procedures

Bacterial strains, media and growth conditions. Bacterial strains were cultured in 1:20 diluted Trypticase Soy Broth (1:20 TSB) at 37 °C shaking at 200 rpm. For solidified medium, TSB was supplemented with 1.5 % agar. The following antibiotics were used: ofloxacin, amikacin and ceftazidime. Concentrations are indicated throughout the text. Strains used in this Chapter are listed in Table 6.1.

Table 6.1: Strains used in this Chapter

Strain	Description[a]	Source or reference
P. aeruginosa PA14	Wild type, Km^R	Lee *et al.* (2006)
P. aeruginosa PAO1	Wild type	Morita *et al.* (2001)
P. aeruginosa YM64	*mexAB-oprM::FRT*, *mexXY::FRT*, *mexCD-oprJ::FRT*, *mexEF-oprN::FRT*	Morita *et al.* (2001)
E. coli TOP10	F^- *mcr*A Δ(*mrr-hsdRMS-mcrBC*) φ80*lacZ*ΔM15 Δ*lacX* 74 *recA1 araD139* Δ(*ara leu*) 7697 *galU galK*	Invitrogen
S. aureus SH1000	Functional *rsbU* derivative of 8325-4 *rsbU*$^+$ (wild type strain cured of prophages)	O'Neill *et al.(2010)*; Horsburgh *et al.* (2002)
S. enterica serovar Typhimurium SL1344	Wild type	Hoiseth & Stocker (1981)

Km^R: kanamycin resistant; Tc^R: tetracycline resistant

Small-molecule library. The small-molecule library (< 500 g mol $^{-1}$) was provided in a dry powder 96-well format by the Centre for Drug Design and Discovery (CD3) of the KU Leuven. These small molecules were selected and purchased from multiple commercial

suppliers. Initial selection of compounds was based on different parameters (Lipinski's rule of five, solubility, toxicophores, frequent hitters, unstable fraction) (Walters *et al.*, 1999; Segers *et al.*, 2011).

Compound storage. Just before first use, plates containing the small molecules were centrifuged (4000 rpm, 5 minutes) after which the compounds were dissolved in 100 % dimethyl sulfoxide (DMSO) to a final concentration of 2-5 mM. Next, plates were sealed and stirred for 10 minutes after which a centrifugation step was carried out (4000 rpm, 5 minutes). After usage, dissolved compounds were stored at - 20 °C in closed bags under N_2-atmosphere in a dark environment.

Screening method. In the optimized protocol, stationary phase cells of *P. aeruginosa* cultured in 1:20 TSB were treated for 5 hours in the wells of a 96-well plate with a combination of ofloxacin and compound. Next, the treated cells were diluted 100-fold into fresh TSB growth medium and incubated in an automated OD plate reader (Bioscreen C, Oy Growth Curves Ab Ltd.). Based on a linear relationship between the number of cells incubated in the automated plate reader and the duration of the lag phase of the resulting growth curve, a first selection of promising small molecules was made. Each 96-well plate was evaluated separately and compounds that gave rise to a value that had less than 5 % probability to occur under the given normal distribution, were selected. The effect of the compound in combination with ofloxacin was re-evaluated independently three times and compared to the effect of treatment with only ofloxacin.

Persistence assay. This assay was carried out as described before (Chapter 4) with minor adaptations. Briefly, *P. aeruginosa* PA14 wild-type cells were grown overnight until stationary phase. Next, cells were treated in a 96-well plate with a combination of antibiotic and compound for 5 hours (unless indicated otherwise). Plates were sealed with a breathable sealing membrane (Greiner Bio-One) and incubated at 37 °C while shaking at 200 rpm. After treatment, cells were washed in 10 mM $MgSO_4$ (6000 rpm, 10 minutes, 4 °C) after which a dilution series was plated out on solid growth medium to determine the number of viable cells.

Statistical analysis. Statistical analysis was carried out as described in Chapter 4 on the log CFU values.

6.3 Results

6.3.1 Optimization of screening method

Previously, a screening method was designed and successfully used to select mutants that display a significant difference in persister fraction after treatment with the antibiotic ofloxacin (De Groote *et al.*, 2009). Based on a linear relationship between the number of surviving persisters and the duration of the lag phase of the surviving population upon reinoculation, mutants that produced an altered number of persister cells were selected. Here, this screening method was optimized for detection of small molecules that, in combination with ofloxacin, significantly reduce the number of persister cells compared to a control treatment.

6.3.1.1 Choice of growth medium, starting culture and antibiotic

Since nutrients are not always abundantly present at the site of infection, *in vivo* situations are likely better represented by poor medium. Additionally, it was reported that small molecules can interact with peptides present in rich growth media (personal communication, CD3), which lowers the active concentration of the compound. Therefore, it was decided to optimize the screening conditions using 1:20 diluted TSB medium.

Fast-growing exponential phase cultures typically contain few or even no persister cells. However, when bacterial cultures enter stationary phase, the persister fraction rises significantly. This growth phase-dependence of the persister fraction has been observed for many bacterial species after treatment with different classes of antibiotics (Spoering & Lewis, 2001; Keren *et al.*, 2004a). The number of persister cells is even higher in stationary phase cultures than in biofilms (Spoering & Lewis, 2001). Since planktonic and biofilm cultures display similar killing kinetics when exposed to a bactericidal agent, it is believed that persister cells present in both populations are similar (Spoering & Lewis, 2001). Therefore, as planktonic populations are easier to manipulate than biofilm cultures, the screening was carried out on free-living stationary phase cells.

In literature, several mechanistically different antibiotics such as ß-lactams, aminoglycosides and fluoroquinolones have been used to study persistence (Spoering & Lewis, 2001).

According to the guide for antibiotic usage published by the University Hospital of Leuven[12], ß-lactam antibiotics are widely recommended for treatment of *P. aeruginosa* infections. However, this class of antibiotics cannot be used in our setup as they only act bactericidal on fast-growing cells (Tuomanen *et al.*, 1986). Furthermore, it was observed that the aminoglycoside tobramycin was ineffective in killing stationary phase cultures (Spoering & Lewis, 2001). Therefore, it was decided to use the fluoroquinolone ofloxacin in our screening. Ofloxacin is a second-generation quinolone, displaying improved activity against DNA gyrase, the main target of quinolones in Gram-negative bacteria. Because of the presence of a fluorine, these second-generation quinolones are often named fluoroquinolones (Aldred *et al.*, 2014).

6.3.1.2 Choice of antibiotic and compound concentration

To find compounds that specifically act on persister cells, conditions were optimized at which only persister cells survive after treatment with ofloxacin. Killing curves were determined using varying antibiotic concentrations. Based on these results (Figure 6.1A) an ofloxacin concentration of 10 µg mL^{-1} was chosen since higher concentrations did not lead to a further reduction of the number of surviving cells. Previously, it was shown in our group that incubation times beyond 5 hours of treatment did not improve bactericidal activity of ofloxacin. Consequently, treatment duration was set at 5 hours.

Typically, whole-cell based screens for new antibacterials or potentiators are carried out using compound concentrations in the micromolar range (Payne *et al.*, 2007; Ejim *et al.*, 2011). Therefore, we decided to screen the library at a final concentration of 20-50 µM.

Since the compounds are dissolved in DMSO, the susceptibility of *P. aeruginosa* cells towards this solvent was tested. A concentration of 2 % (v/v) did not affect cell growth, whereas a concentration of 4 % or higher acts growth inhibitory. Complete growth inhibition was achieved at a concentration of 16 % (Figure 6.1B). Based on the stock solution concentration and the choice of compound concentration used in the screening, a DMSO background concentration of 1 % will be reached, which does not affect growth.

[12] http://www.uzleuven.be/antibioticagids/

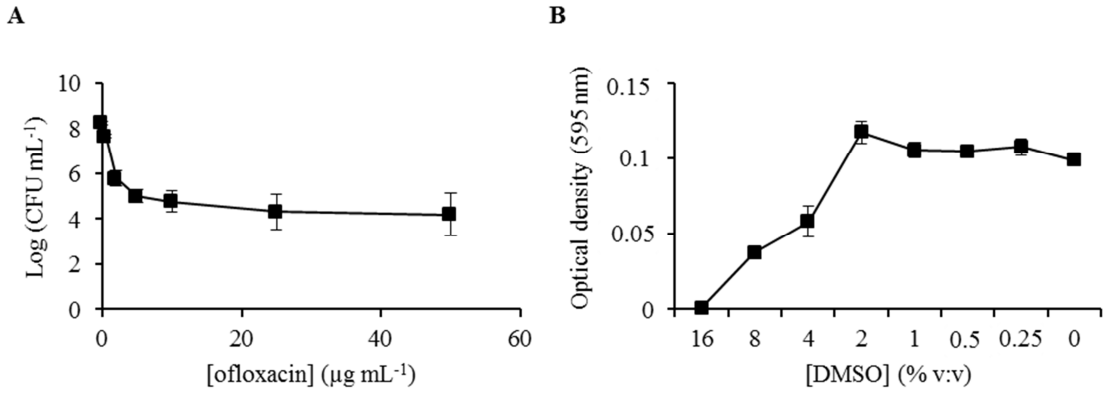


Figure 6.1: Optimization of screening conditions. A. Stationary phase cells were treated for 5 hours with different concentrations of ofloxacin prior to viable cell counts by plating**. B.** Susceptibility of growing cells to DMSO was determined by exposing growing cells to a range of DMSO concentrations (v:v). Effect on growth was evaluated by determination of OD_{595}. Each experiment was repeated independently at least three times, data points correspond to the mean. Error bars represent standard error of the mean (SEM).

6.3.1.3 Correlation between number of cells and lag phase

To define the linear correlation between the number of cells incubated in the automated plate reader and the lag phase of the resulting growth curve, the following experiment was carried out: *P. aeruginosa* was grown in 1:20 TSB until stationary phase after which the cells were treated with water or ofloxacin (10 µg mL^{-1}) for 5 hours. After treatment, a 10-fold dilution series was prepared in 10 mM $MgSO_4$. Next, these samples were diluted 100-fold into fresh TSB growth medium and incubated in the automated plate reader to generate growth curves. In parallel, an aliquot of each diluted sample was plated on solid growth medium in order to determine the number of viable cells present in each sample. This experiment was repeated independently three times. The results along with an example are shown in Figure 6.2.

6.3.2 Screening of a small-molecule library for anti-persister compounds

A small-molecule collection comprising 23909 commercially available diverse molecules was screened to identify compounds that, in combination with the fluoroquinolone antibiotic ofloxacin, reduce the persister fraction compared to treatment with only ofloxacin. The screening is based on a correlation between the number of surviving persisters and the duration of the lag phase of the surviving population (De Groote *et al*., 2009).

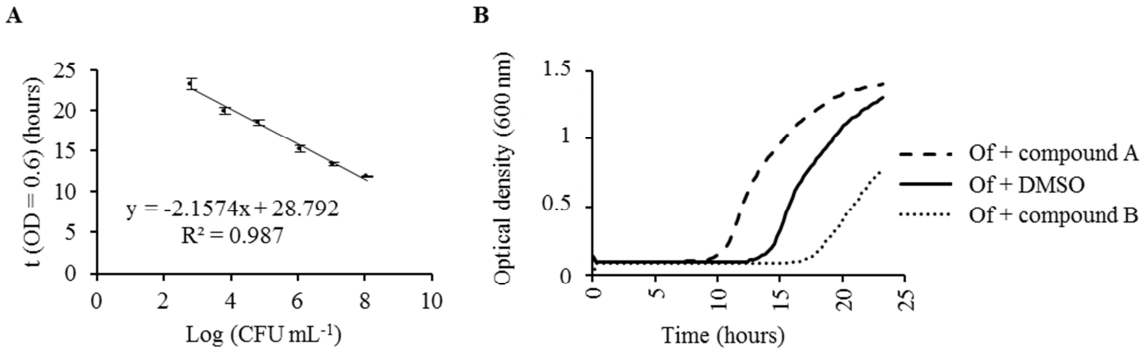


Figure 6.2: Selection method of promising anti-persister compounds. A. Linear relationship between the logarithmic value of the number of cells incubated in the automated plate reader and the duration of the lag phase of the resulting growth curves, represented by the time needed to reach an optical density (595 nm) of 0.6. Values represent the mean of 3 independent repeats, error bars indicate standard deviation. **B.** Representative growth curves of cells diluted after treatment with a combination of ofloxacin (Of) and compound A (dashed curve), compound B (dotted curve) or DMSO (solid curve). Compound A increases and compound B reduces the number of cells in combination with ofloxacin as compared to monotreatment, respectively.

Based on this screening, 105 compounds were selected and re-evaluated as described in Experimental procedures. The majority of them (85.7 %) caused no or only a minor effect on the persister fraction in combination with ofloxacin as compared to monotreatment with the antibiotic. The remaining compounds, in total 15 out of 105, decreased the number of surviving persister cells over four times compared to treatment with only ofloxacin (Figure 6.3A) and were selected for independent confirmation experiments. The effect of the 15 selected compounds was independently evaluated using plate counts. Here, cells were plated out after treatment in order to determine the number of viable cells.

Based on these results, five compounds belonging to three structurally distinct families named CIM007228, CIM006251 and CIM010682, were selected (Figure 6.3B). Although the decrease in persister fraction was not always statistically significant, we decided to test a resupply for three of these compounds, each of them belonging to a different compound family. Again, the persister fraction was not significantly reduced but a preliminary test showed that doubling the tested concentration further decreased the persister fraction (data not shown). Therefore, it was decided to evaluate commercially available analogues of these three compound families in a dose-response study.

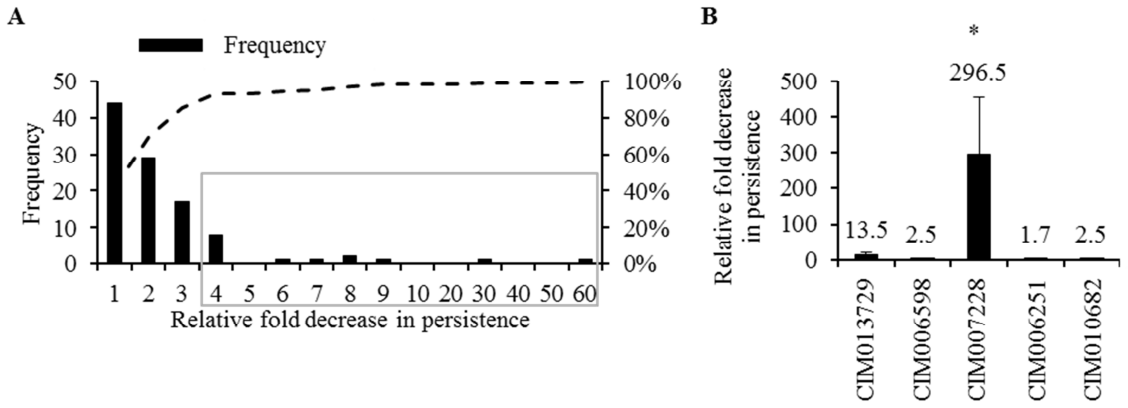


Figure 6.3: Overview of compounds selected during screening. A. Selection of 15 compounds after re-evaluation of the initially selected compounds. Treated cells were diluted 100-fold after which growth was monitored. Based on the correlation between the number of surviving cells and duration of the lag phase of the resulting growth curve, the relative decrease in persistence was calculated. 15 compounds, enclosed in the grey box, were selected for confirmation experiments. **B.** Relative decrease in persistence compared to treatment with only ofloxacin. Stationary phase cells were treated for 5 hours with a combination of ofloxacin and compound after which the number of remaining viable cells was determined. Data points correspond to the mean of three independent experiments, error bars represent SEM. Statistical analysis was carried out on the number of surviving cells. * $P < 0.0001$.

6.3.3 Evaluation of commercially available analogues

6.3.3.1 Dose-response analysis

Evaluation of chemical analogues by monitoring regrowth after treatment

44 commercially available analogues of the three identified structural families were obtained and a first dose-response experiment was conducted. Stationary phase cells were treated for 5 hours at 37 °C with a combination of ofloxacin (10 µg mL^{-1}) and compound. For each analogue, a concentration gradient was tested, with the highest concentration being 200 µM and the lowest 0.78 µM. The background concentration of DMSO was kept constant at 1 % (v/v). After treatment, samples were diluted and incubated in the automated plate reader. Based on the duration of the lag phase of the surviving population, the difference in number of surviving cells was calculated. 10 out of 44 chemical compounds tested showed a dose-response effect (Table 6.2).

Table 6.2: Overview of chemical analogues of the three compound families. The compounds are classified as those possessing and those lacking an anti-persistence dose-response effect as determined by monitoring regrowth of diluted cells after treatment.

Family 1 (CIM007228)		Family 2 (CIM006251)		Family 3 (CIM010682)	
Dose response	**No dose response**	**Dose response**	**No dose response**	**No dose response**	
CIM007228	CIM008379	CIM006251	CIM056264	CIM009198	CIM056287
CIM013729	CIM008494	CIM056262	CIM056265	CIM009279	CIM056288
CIM056268	CIM056263		CIM056277	CIM009682	CIM056289
CIM056271	CIM056269		CIM056278	CIM010582	CIM056290
CIM056282	CIM056270			CIM011277	CIM056291
CIM056283*	CIM056272			CIM011495	CIM056292
CIM056305	CIM056273			CIM056284	CIM056293
CIM056307	CIM056274			CIM056285	CIM056294
	CIM056275			CIM056286	
	CIM056276				
	CIM056306				
	CIM056308				
	CIM056309				

*Preliminary tests showed a bactericidal effect

The objective of this study was to identify compounds that specifically target persister cells. In this way it is possible, by unraveling the compounds' mode of action, to gain more insight in the fundamentals underlying these dormant antibiotic-insensitive cells. Therefore, a preliminary test was carried out in which the bactericidal effect of all analogues was evaluated. Here, stationary phase cells were treated for 5 hours with compound (concentration range of 0-200 μM) after which the treated cells were diluted in fresh growth medium to monitor regrowth. Based on the duration of the lag phase, the number of viable cells was determined. One of the 10 selected compounds, CIM056283, showed bactericidal activity and therefore was discarded for further dose-response analysis. For the remaining 9 compounds, the effect on persistence is shown in Figure 6.4. Preliminary data on bactericidal activity for all analogues can be found in Appendix B (Figure B.1), as well as data on the anti-persistence effect of the 35 non-selected compounds (Figure B.2).

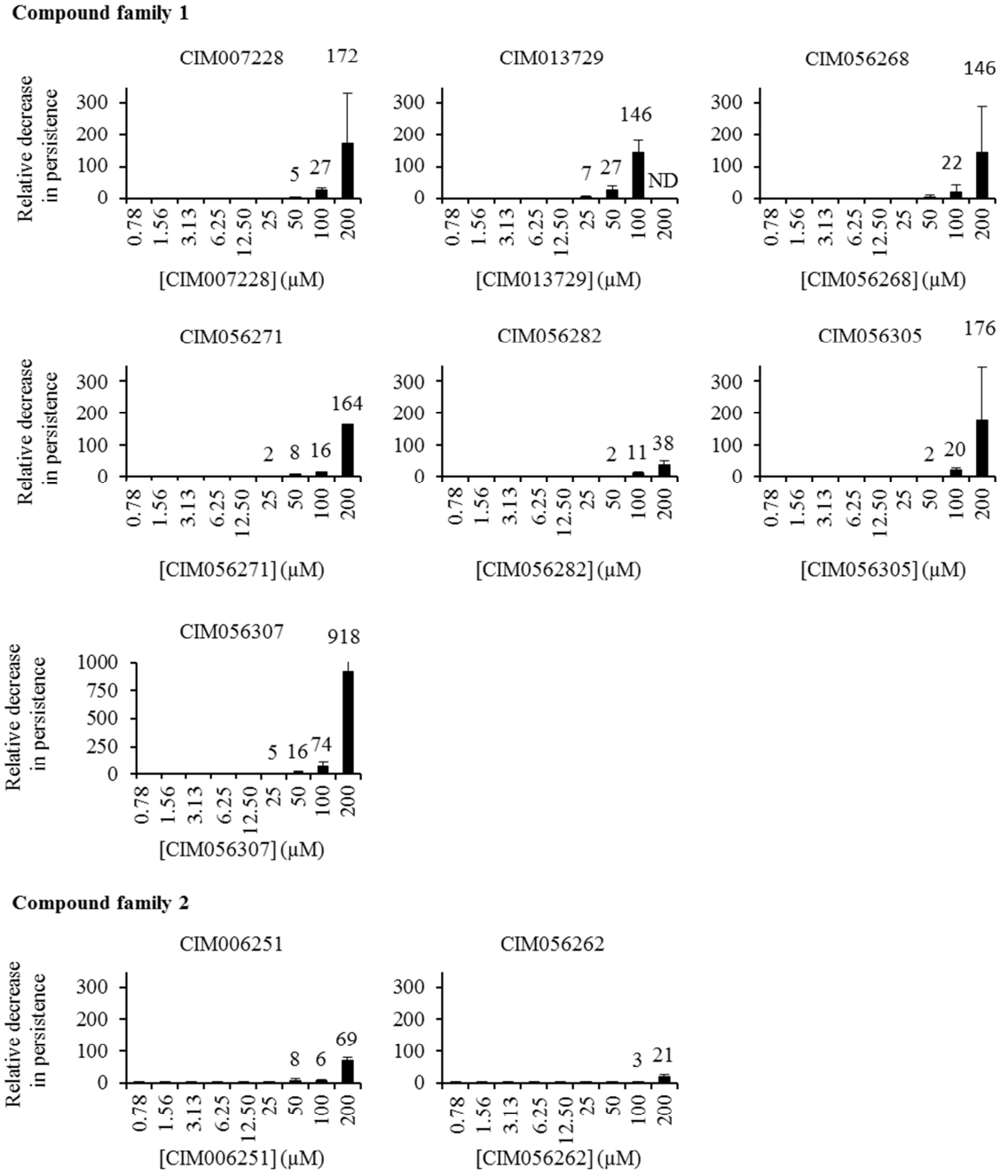


Figure 6.4: Dose-response analysis of selected commercially available analogues based on the duration of the lag phase. Stationary phase cells were treated for 5 hours with a combination of ofloxacin (10 µg mL^{-1}) and compound (0.78 – 200 µM). Next, samples were 100-fold diluted into fresh TSB growth medium and growth was monitored. Based on the duration of the lag phase of each individual growth curve, the number of surviving cells was estimated. Data points represent the average relative reduction in number of cells (indicated above selected data points) compared to treatment with only ofloxacin. The experiment was independently repeated three times, error bars represent SEM. ND, not detectable.

7 of these 9 selected compounds belong to compound family 1. In general, an effect is visible when ofloxacin is combined with 25, 50 or 100 µM of anti-persister molecule, depending on the tested compound, but a particular better effect is observed when ofloxacin is combined with 200 µM compound. For some molecules, like CIM007228, CIM056268 and CIM056305, quite some variation is seen between the repetitions of the independent experiments. The best performing compound of family 1 is CIM013729. Combination of 100 µM of CIM013729 with ofloxacin resulted in a 146-fold decrease of the persister fraction compared to treatment with only ofloxacin. When 200 µM of this compound is used, regrowth of the surviving population was not detected within 24 hours. Compound CIM056282 displays the lowest effect on the persister fraction, resulting in a 38-fold reduction in persister cells when 200 µM is combined with the antibiotic ofloxacin. The two remaining compounds both belong to compound family 2, with CIM006251 displaying the biggest effect. Combination of 200 µM CIM006251 with ofloxacin decreased the persister fraction 69-fold. No compounds of family 3 showed a dose-response effect on persistence.

Further analysis of selected analogues by viable cell counting

The effect of the nine selected compounds in combination with ofloxacin was independently tested with plate count experiments (Figure 6.5). In general, compounds that displayed the largest effect when evaluated by monitoring the duration of the lag phase were confirmed by plate counting experiments. For compound family 1, CIM007228, CIM056271, CIM056305 and CIM056307 caused a significant decrease of the persister fraction in combination with ofloxacin when administered at a concentration of 200 µM. CIM056282, the compound displaying the weakest effect in the initial dose-response experiment, was not confirmed by plate counting experiments. Remarkably, the effect of CIM013729 and CIM056268 in combination with ofloxacin was not confirmed. For compound family 2, CIM006251, although not statistically significant, caused a 35-fold reduction of the persister fraction upon combination with ofloxacin. As was the case in the previous experiment, combination of ofloxacin and 200 µM of compound drastically reduces the number of cells, while no or a remarkably lower effect is detected upon use of lower compound concentrations. Based on these data, it seems that a certain threshold level (> 100 µM) of compound is needed to establish the desired effect. In addition, preliminary plate counting tests were carried out to

assess the bactericidal effect (see Appendix B, Figure B.3). Based on these data, it seems that CIM007228, CIM056271 and CIM056305 possibly exert a minor bactericidal effect when applied at 200 µM. However, additional tests have to be carried out to confirm these results.

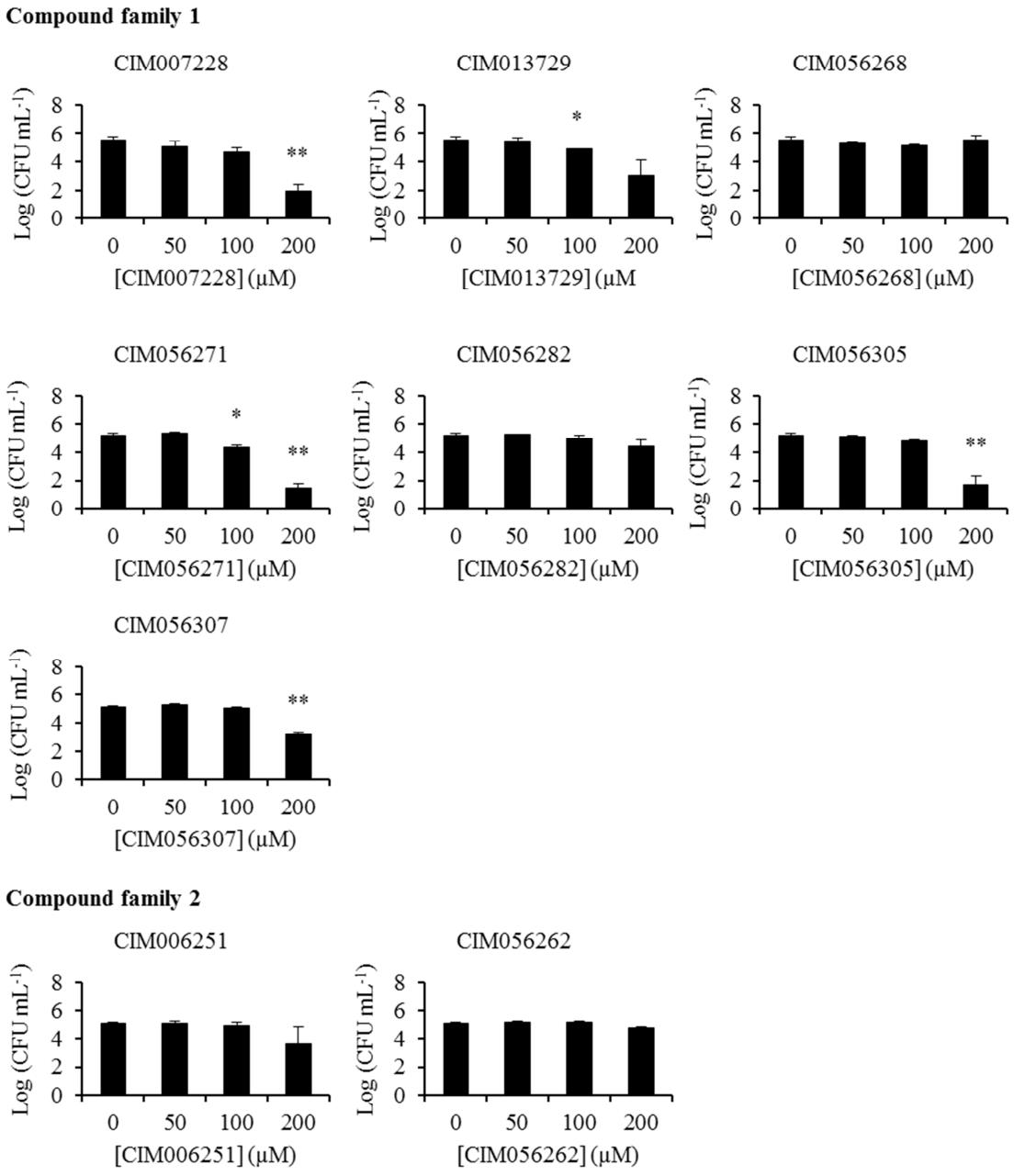


Figure 6.5: Dose-response analysis of selected commercially available analogues using plate counts. Stationary phase cells were treated for 5 hours with a combination of ofloxacin (10 µg mL^{-1}) and compound (0 – 200 µM). Next, samples were diluted and plated on solid growth medium in order to determine the number of viable cells. Data points represent the average of three independent experiments, error bars represent SEM. The relative decrease in viable cells is indicated above selected data points. *P < 0.05, **P < 0.005.

Evaluation of the anti-persistence effect by viable cell counting revealed that assessment by monitoring regrowth of treated cells underestimated the reduction in persister fraction. For example, according to the correlation between the number of surviving cells and the lag phase, CIM056271 reduced the persister fraction 164 times as compared to treatment with only ofloxacin. However, when the effect was determined by plate counting experiments, a ~5700 fold reduction was observed. Similar differences were observed for CIM007228 and CIM056305. On the other hand, CIM056307 displayed a smaller effect upon evaluation by viable cell counting. For some compounds such as CIM056268, no correlation was visible between the two independent experimental setups. Despite the fact that a clear reduction in persistence was detectable upon monitoring regrowth of treated cells, this effect was not confirmed by plate counting experiments. This suggests that CIM056268 specifically affects regrowth of the surviving cells instead of reducing the number of viable cells during treatment. Based on these observations, we decided to analyze the structure activity relationship (SAR) by using plate counting experiments. As the effect on the persister fraction determined by viable cell counting is most clear when using 200 µM of compound, SAR analysis will be carried out using only this compound concentration.

6.3.3.2 Structure-activity relationship analysis

The anti-persistence effect was evaluated by determining the number of viable cells after a 5 h treatment with ofloxacin combined with each analogue (200 µM). The bactericidal effect of each chemical analogue was evaluated by treating stationary phase cells for 5 hours with only the compound. Four additional analogues (CIM057662, CIM057663, CIM057668 and CIM057669), belonging to compound family 1, were provided by CD3 and were taken along in the SAR analysis.

SAR analysis of compound family 1 (Figure 6.6) revealed seven compounds that significantly reduced the persister fraction compared to ofloxacin treatment. Three of them, CIM056272, CIM056283 and CIM007228, also exhibited a significant bactericidal effect on stationary phase cells. For the reason stated above, these compounds were excluded from further analysis. Of the four remaining compounds, CIM056271, further referred to as SPI001, was selected for further characterization. This compound reduces the persister fraction ~7200-fold ($P < 0.0001$) compared to treatment with only ofloxacin.

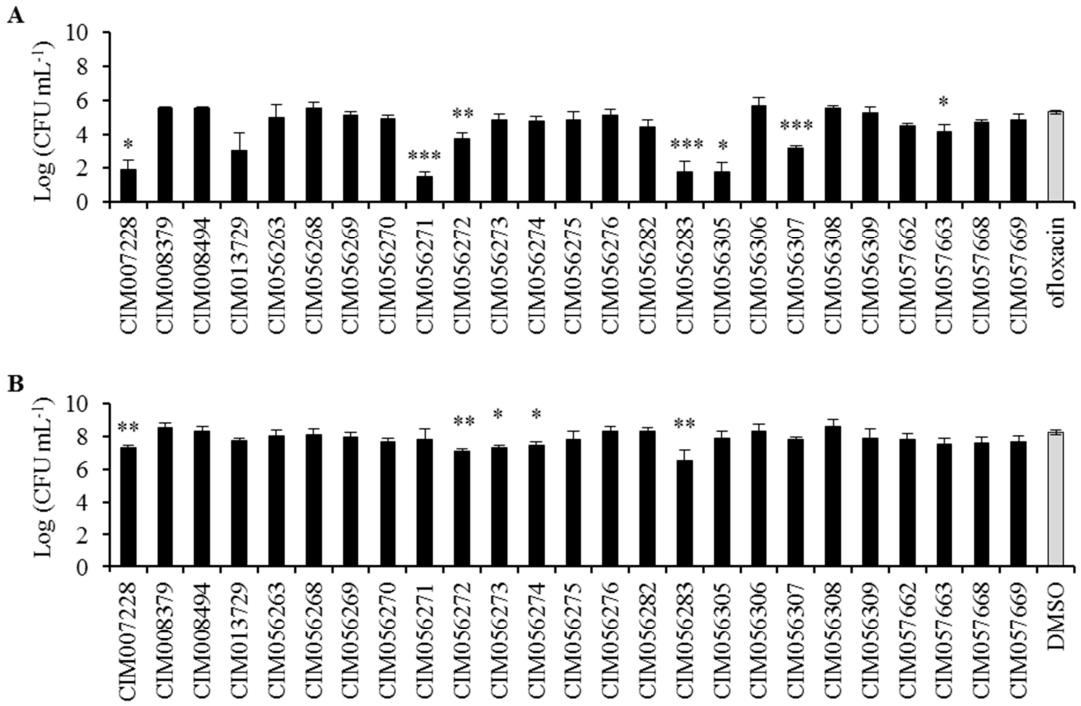


Figure 6.6: Structure activity analysis of compound family 1. A. Evaluation of the anti-persistence effect of the chemical analogues. Stationary phase cells were treated for 5 hours with a combination of ofloxacin (10 µg mL^{-1}) and compound (200 µM). As a control, cells were treated with ofloxacin combined with DMSO (1 % v/v) represented by the light grey bar. **B.** Evaluation of the bactericidal effect of each chemical analogue. Stationary phase cells were treated for 5 hours with the chemical analogue (200 µM), as a control cells were treated with the solvent DMSO (1 % v/v). After treatment, cells were washed and plated on solid growth medium to determine the number of viable cells. Data points represent the average of three independent repeats, error bars correspond to SEM. *P < 0.05, **P < 0.005 and *** P < 0.0001.

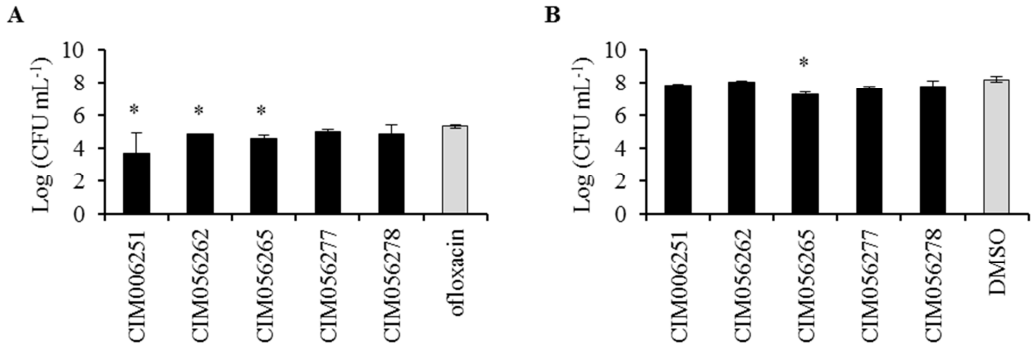


Figure 6.7: Structure activity analysis of compound family 2. A. Evaluation of the anti-persistence effect of the chemical analogues. Stationary phase cells were treated for 5 hours with a combination of ofloxacin (10 µg mL^{-1}) and compound (200 µM). As a control, cells were treated with ofloxacin combined with DMSO (1 % v/v) represented by the light grey bar. **B.** Evaluation of the bactericidal effect of each chemical analogue. Stationary phase cells were treated for 5 hours with the chemical analogue (200 µM), as a control cells were treated with the solvent DMSO (1 % v/v). After treatment, cells were washed and plated on solid medium to determine the number of viable cells. Data points represent the average of three independent repeats, error bars correspond to SEM. *P < 0.05.

Based on SAR analysis of compound family 2 (Figure 6.7), three compounds were selected that significantly decreased the persister fraction compared to ofloxacin treatment. Since two of them also acted bactericidal, the remaining compound CIM006251 was selected for further analysis. This compound decreased the persister fraction up to ~45 fold ($P < 0.05$). For convenience, this compound was named SPI002.

Analysis of all available chemical analogues of compound family 3 (Figure 6.8) resulted in the identification of two compounds, CIM010582 and CIM011495, that significantly reduced the persister fraction compared to ofloxacin treatment (~6 and ~8 fold respectively). Unfortunately, these two compounds also acted slightly bactericidal ($P < 0.05$) as compared to ofloxacin and were therefore excluded from further analysis.

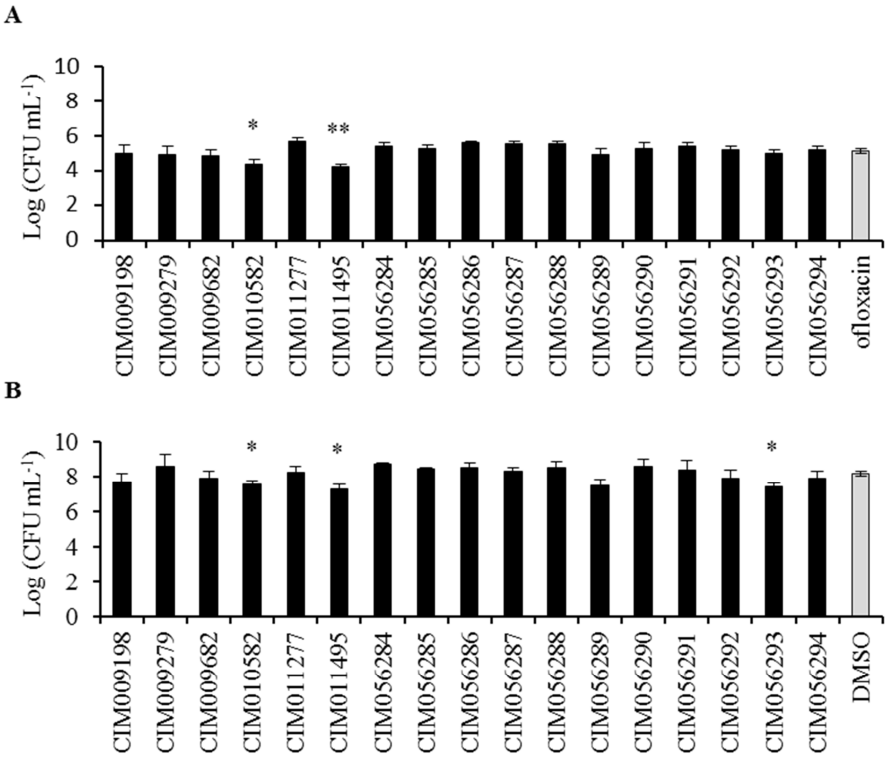


Figure 6.8: Structure activity analysis of compound family 3. A. Evaluation of the anti-persistence effect of the chemical analogues. Stationary phase cells were treated for 5 hours with a combination of ofloxacin (10 µg mL^{-1}) and compound (200 µM). As a control, cells were treated with ofloxacin combined with DMSO (1 % v/v) represented by the light grey bar. **B.** Evaluation of the bactericidal effect of each chemical analogue. Stationary phase cells were treated for 5 hours with the chemical analogue (200 µM). As a control cells were treated with the solvent DMSO (1 % v/v). After treatment, cells were washed and plated on solid medium to determine the number of viable cells. Data points represent the average of three independent repeats, error bars correspond to SEM. *$P < 0.05$ and ** $P < 0.005$.

6.3.4 Further characterization of SPI001 and SPI002

6.3.4.1 Combination with other antibiotics

During the screening, small molecules were combined with ofloxacin. This antibiotic belongs to the fluoroquinolones, which target DNA replication (Aldred *et al.*, 2014). Other widely used antibiotics in clinical settings against *P. aeruginosa* infections include the aminoglycoside amikacin and the cephalosporin ceftazidime[13]. Therefore the effect of SPI001 and SPI002 was evaluated in combination with these antibiotics.

Optimization of a persistence assay with amikacin and ceftazidime

To assess the effect on persister cells, the concentration of antibiotic at which only persister cells survive was determined. *P. aeruginosa* stationary phase cells were subjected to a range of amikacin concentrations (0 - 200 µg mL^{-1}, minimal inhibitory concentration (MIC) value = 0.2 µg mL^{-1}) during 5 hours. After treatment, cells were washed, diluted and plated on solid growth medium. This experiment was independently repeated three times. Based on these results, no significant additional killing occurs when cells are treated with 50 µg mL^{-1} or higher concentrations of amikacin (Figure 6.9A). This indicates the presence of an amikacin-tolerant persister fraction. Subsequent persistence assays were carried out using 100 µg mL^{-1} amikacin.

Ceftazidime belongs to the ß-lactam antibiotics and therefore has no bactericidal effect on stationary phase cells. In order to find the most suitable conditions to obtain a stable persister fraction, the following experiment was carried out. *P. aeruginosa* stationary phase cells were diluted 100-fold into fresh 1:20 TSB. When the culture reached an optical density of approximately 0.005, 0.02, 0.050 and 0.125, the cells were challenged with different concentrations of ceftazidime (0 - 60 µg mL^{-1}, MIC value = 6.25 µg mL^{-1}). After 5 hours of treatment at 37 °C, cells were washed, diluted and plated on solid growth medium in order to determine the number of surviving cells. This experiment was repeated independently three times. A stable persister fraction was observed, regardless at which optical density the treatment was initiated (Figure 6.9B). Remarkably, the persister fraction already started

[13] http://www.uzleuven.be/antibioticagids/

increasing when cells entered exponential phase (OD_{595} = 0.05). This is not in accordance with earlier reports in literature, where the persister fraction only starts rising when cells enter mid-late exponential phase (Keren *et al.*, 2004a). For subsequent persistence experiments, growing cells that reached an optical density of 0.02 were treated with 30 µg mL^{-1} ceftazidime.

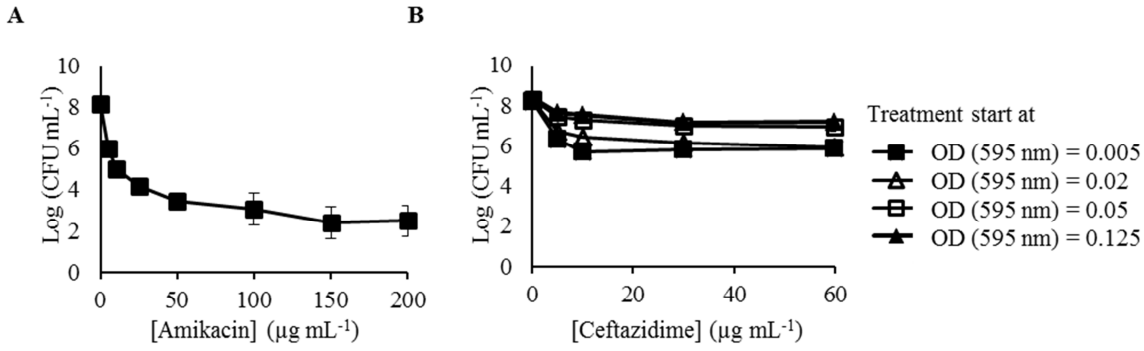


Figure 6.9: Optimization of persistence assays for amikacin and ceftazidime. A. Stationary phase cells were treated for 5 hours with different concentrations of amikacin prior to viable cell counts by plating. **B.** Stationary phase cells were diluted into fresh medium and after 1, 2, 3 and 4 hours respectively, growing cells were treated with increasing concentrations of ceftazidime. Viable cell counts were determined by plating. Data points correspond to the mean. Each experiment was repeated independently at least three times. Error bars represent SEM.

Effect of SPI001 and SPI002 in combination with amikacin or ceftazidime

Stationary phase cells were treated for 5 hours with amikacin (100 µg mL^{-1}) combined with either SPI001 or SPI002 (200 µM). Growing cells were treated with ceftazidime (30 µg mL^{-1}) and one of the two compounds. Since exponential phase cells are typically more sensitive towards antibiotic treatment, different concentrations of SPI001 and SPI002 were tested (0-200 µM). The results are shown in Figure 6.10. SPI002 significantly reduces the persister fraction in combination with ceftazidime and amikacin. SPI001, on the other hand, completely eliminates the bacterial population in combination with both antibiotics when a concentration of 200 µM is used. Combining lower concentrations of compound with ceftazidime did not result in a significant reduction of the persister fraction (Figure 6.10B).

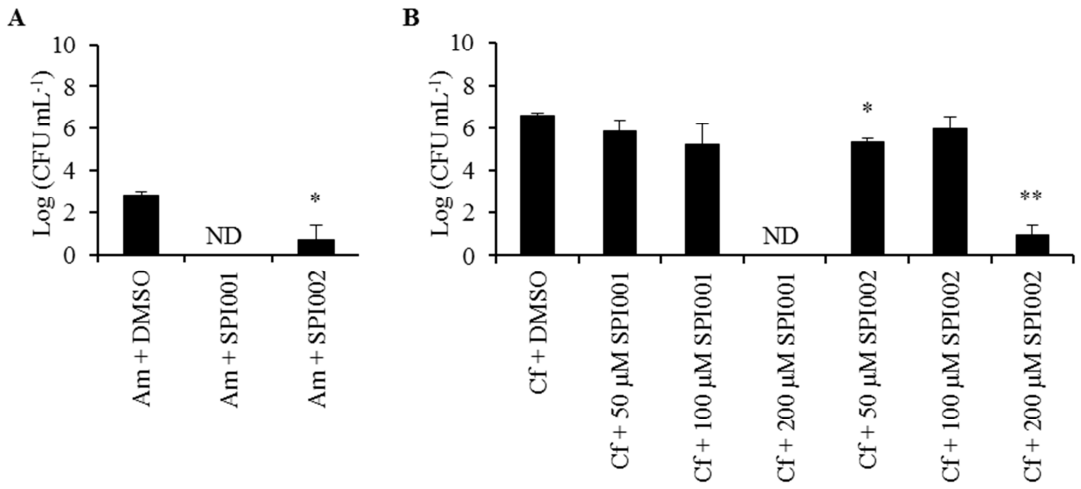


Figure 6.10: Effect of molecules in combination with amikacin and ceftazidime on *P. aeruginosa*. A. Stationary phase cells and **B.** exponential phase cells of *P. aeruginosa* were treated for 5 hours with amikacin (Am, 100 µg mL^{-1}) or ceftazdime (Cf, 30 µg mL^{-1}), respectively, in combination with 1 % DMSO, 200 µM SPI001 or 200 µM SPI002. Data points correspond to the mean relative persister fraction. Each experiment was repeated independently at least three times. Error bars represent SEM. * $P < 0.05$, ** $P < 0.0005$. ND, not detectable.

Above, we demonstrated that addition of only SPI001 or SPI002 to *P. aeruginosa* stationary phase cells did not cause a significant bactericidal effect (see Figure 6.6B and Figure 6.7B). However, treating growing cells with SPI001 or SPI002 significantly affects viability as shown in Figure 6.11. Both SPI001 and SPI002 exert a significant bactericidal effect when administered at a concentration of 100 µM or higher, while SPI002 already kills *P. aeruginosa* cells at 50 µM. These results indicate that SPI001 and SPI002 act bactericidal on growing cells but not on stationary phase cells of *P. aeruginosa*. Moreover, compounds SPI001 and SPI002 are more potent than ceftazidime, reducing the number of viable cells to 5.4×10^2 and 1.4×10^3 cells respectively when administered at 200 µM. However, the combination of ceftazidime with SPI001 or SPI002 shows a better effect than each of the treatments separately.

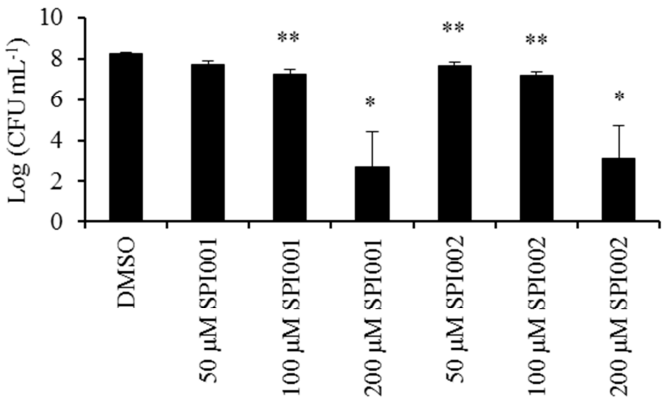


Figure 6.11: Effect of SPI001 and SPI002 on growing cells. The anti-persistence (combination of ofloxacin and compound) and bactericidal effect of SPI001 and SPI002 was determined by treating growing cells for 5 hours. After treatment, cells were washed, diluted and the number of viable cells was determined by means of plate counting. Data points represent the average of three independent repeats, errors bars indicate SEM. *P < 0.1, **P < 0.05.

6.3.4.2 Effect on other pathogens

Persister cells have been suggested to play an important role in the recalcitrant nature of infections caused by *P. aeruginosa* but also by several other pathogens (Cohen *et al.*, 2013). We therefore examined whether SPI001 or SPI002 are able to reduce the persister fraction of the Gram-negative pathogens *E. coli* and *Salmonella* Typhimurium and the Gram-positive bacterium *Staphylococcus aureus*. For each bacterium, the anti-persistence and bactericidal effect of SPI001 and SPI002 was evaluated. The anti-persistence effect is determined by treating stationary phase cells for 5 hours with a combination of antibiotic and compound. The bactericidal effect is examined by treating stationary phase cultures for 5 hours with the anti-persister compound only. After treatment, cells were washed, diluted and plated on solid growth medium to determine the number of surviving cells.

Effect on *Escherichia coli*

The anti-persistence and bactericidal effect of SPI001 and SPI002 was tested on *E. coli*, a model organism for studying persistence. Previous optimization experiments carried out in our research group indicate that the use of 5 µg mL^{-1} ofloxacin for 5 hours allows survival of persister cells only (Verstraeten, 2011). Therefore, to assess the anti-persistence effect,

stationary phase cells were treated with the combination of ofloxacin and compound. As is shown in Figure 6.12A, SPI001 and SPI002 are both capable of reducing the persister fraction in combination with ofloxacin. SPI001 shows the best effect: combining ofloxacin with 50 µM reduces the persister fraction 37-fold while addition of 100 µM completely eliminated the bacterial population. Treatment of stationary phase cells with the combination ofloxacin – SPI002 (100 µM) reduced the persister fraction almost 4000 times. In addition, SPI001 and SPI002 both act bactericidal on stationary phase *E. coli* cells (Figure 6.12B). Upon administration of 100 µM of SPI002, the number of viable cells decreased almost 8000-fold, whereas treatment with 100 µM of SPI001 completely eliminated the entire bacterial population.

Effect on *Salmonella enterica* serovar Typhimurium

Salmonella is an enteric pathogen, responsible for the outbreak of severe food-borne infections worldwide (Krtinic *et al.*, 2010). This bacterium can enter and replicate within macrophages (Haraga *et al.*, 2008), hereby evading the host immune system. Recently it was published that, immediately after uptake into these macrophages, *Salmonella* forms a non-replicating population representing antibiotic-insensitive persister cells (Helaine *et al.*, 2014). In this way, persisters form a reservoir of bacteria that can cause relapse of infection. Therefore, we tested the susceptibility of this pathogen to the anti-persistence and bactericidal effect of SPI001 and SPI002.

Previous optimization experiments carried out in our group indicate that treatment of 5 hours using 5 µg mL^{-1} ofloxacin results in a stable persister fraction (Valerie Defraine, personal communication). Therefore, compounds were combined with ofloxacin to assess the anti-persistence effect. Assessment of the bactericidal effect was carried out by Valerie Defraine. As is shown in Figure 6.13A, both anti-persister molecules have an effect on the persister fraction when applied at a concentration of 100 µM or higher. However, in this case, the best effect was obtained with the combination ofloxacin and SPI002. The combination of ofloxacin with 100 µM SPI002 reduced the persister fraction 450-fold compared to ofloxacin treatment, while using a concentration of 200 µM reduced the number of persisters more than 4 log units. Furthermore, as was the case in *E. coli*, SPI001 and SPI002 both act bactericidal on stationary phase cultures of *Salmonella* (Figure 6.13B). Addition of 100 µM drastically

reduced the number of surviving cells (> 5 log units), treating cells with 200 µM SPI001 even completely eliminated all detectable viable cells.

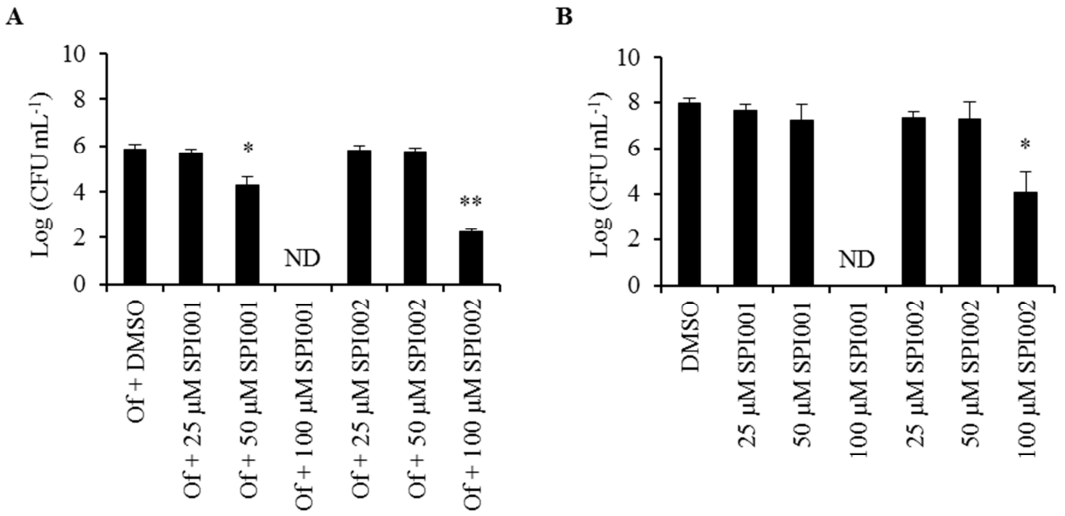


Figure 6.12: Effect of SPI001 and SPI002 on *E. coli*. A. Anti-persistence and **B.** bactericidal effect was evaluated by treating stationary phase cultures for 5 hours with a combination of ofloxacin (Of) and compound or the compound alone, respectively. After treatment, cells were washed, diluted and plated on solid medium to determine the number of surviving cells. Data points represent the average of three independent repeats, error bars indicate SEM. *P < 0.05 and **P < 0.0001. ND, not detectable.

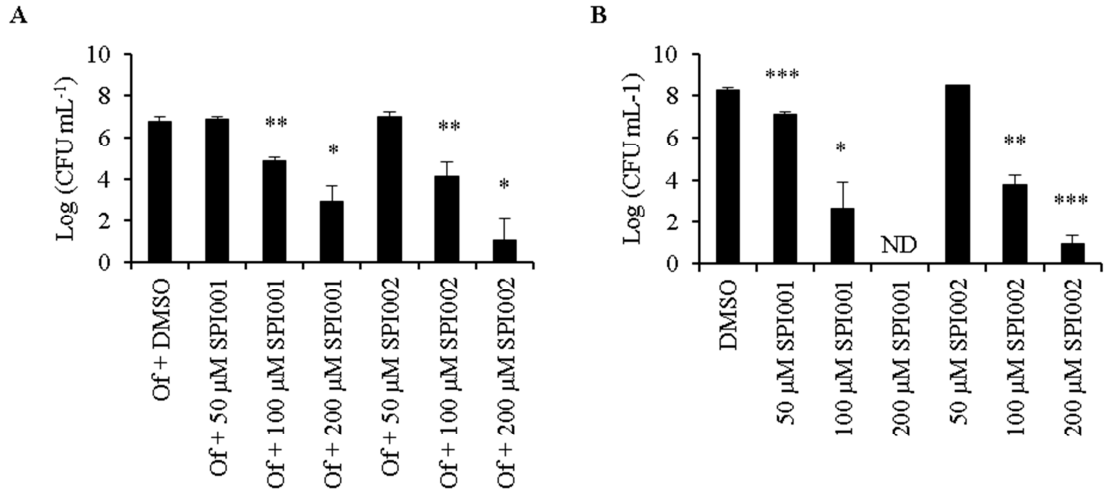


Figure 6.13: Effect of SPI001 and SPI002 on *S.* Typhimurium. A. Anti-persistence and **B.** bactericidal effect was evaluated by treating stationary phase cultures for 5 hours with a combination of ofloxacin (Of) and compound or the compound alone, respectively. After treatment, cells were washed, diluted and plated on solid medium to determine the number of surviving cells. Data points represent the average of three independent repeats, error bars indicate SEM. Assessment of the bactericidal effect of SPI001 was carried out by Valerie Defraine. *P < 0.05, **P < 0.005 and ***P < 0.0005. ND, not detectable.

Effect on *Staphylococcus aureus*

The Gram-positive bacterium *S. aureus* is the second most frequently isolated pathogen among all hospital-associated infections, and moreover, is the leading cause of surgical site infections in Europe and throughout the US (ECDC, 2013; Magill *et al.*, 2014). Infections caused by this pathogen are often difficult to eradicate. Exactly 70 years ago, Joseph Bigger discovered the presence of a small antibiotic-insensitive subpopulation of staphylococcal cells that survived antibiotic treatment which he named 'persisters' (Bigger, 1944). Today, these persister cells are believed to play a major role in the recalcitrant nature of *Staphylococcus* infections.

To evaluate the anti-persistence effect, experimental conditions were optimized in our research group. The use of 10 µg mL^{-1} rifampicin for 5 hours killed all antibiotic-sensitive cells, leaving only the persister cells (Valerie Defraine, personal communication). Therefore, the anti-persistence effect of the compounds was examined in combination with rifampicin. The bactericidal effect on stationary phase cultures was assessed by Valerie Defraine. The results are given in Figure 6.14A. A combination of rifampicin with 100 µM anti-persister compound or higher significantly decreases the persister fraction. As was the case for *E. coli* and *P. aeruginosa*, SPI001 causes the biggest decrease of the persister fraction. Combination of rifampicin with 200 µM SPI001 resulted in a 2468-fold reduction. Both anti-persister molecules also act bactericidal on *S. aureus* stationary phase cells (Figure 6.14B). Treatment with 100 µM or higher significantly reduced the number of viable cells. Remarkably, monotreatment with only the persister compound performs better than the combination therapy.

6.3.4.3 Effect on a P. aeruginosa efflux mutant

A higher concentration of SPI001 or SPI002 is needed to significantly decrease the persister fraction in *P. aeruginosa* as compared to *E. coli*. It is known that *P. aeruginosa* has a highly impermeable membrane, 100 times less permeable than *E. coli* (Yoshimura & Nikaido, 1982) (see Section 1.3.1.1). The presence of efflux pumps further prevents the accumulation of molecules in the bacterial cell. To test if SPI001 and SPI002 work more efficiently if more compound is retained within the bacterium, a *P. aeruginosa* efflux mutant was used. This

PAO1-derived mutant strain YM64 lacks four major *mex* operons (*mexAB-oprM*, *mexCD-oprJ*, *mexEF-oprN* and *mexXY*) for multidrug efflux pumps (Morita *et al.*, 2001).

Optimization of a persistence assay with ofloxacin

To determine the concentration of ofloxacin at which only persister cells survive, *P. aeruginosa* wild type and mutant strain YM64 stationary phase cells were treated with increasing concentrations of ofloxacin (0-50 µg mL^{-1}, MIC value = 0.08 µg mL^{-1}) for 5 hours. Based on viable cell counting, no additional killing was observed when using 10 or 5 µg mL^{-1} or higher (Figure 6.15) on wild type and mutant strain respectively. This indicates the presence of an ofloxacin-tolerant persister fraction. To compare the persister fractions of both strains, assessment of the persister fraction will be carried out using 15 µg mL^{-1} ofloxacin.

Effect of SPI001 and SPI002 on the efflux mutant YM64

Both the anti-persistence and bactericidal effect of SPI001 and SPI002 was tested on YM64 and its parental PAO1 wild-type strain. The results show that the efflux mutant was far more sensitive towards the persistence effect of both SPI001 and SPI002, with SPI001 causing the best effect (Figure 6.16). While the persister fraction of the wild-type strain was not affected, combination of ofloxacin with 50 µM SPI001 or higher significantly reduced the persister fraction of the efflux mutant YM64. The combination of SPI002 (100 µM) with ofloxacin reduced the persister fraction of the efflux mutant 385-fold, while no effect was detected upon treatment of the wild type strain. This is in agreement with previous described data where a reduction in persister fraction of PA14 wild type strain was only achieved when using 200 µM of the anti-persister molecules (see Section 6.3.3).

When subjecting the efflux mutant to a treatment with SPI001 alone, a significant reduction in the number of viable cells was detected when using 50 µM or higher. Remarkably, SPI001 and SPI002, when administered at 100 µM, were as potent as ofloxacin on stationary phase cells of the efflux mutant. When the same treatment was performed on the wild-type strain, no decrease was visible at all. Taken together, these data show that, because of the low

permeability of wild-type *P. aeruginosa* cells, more compound is needed to achieve the desired effect.

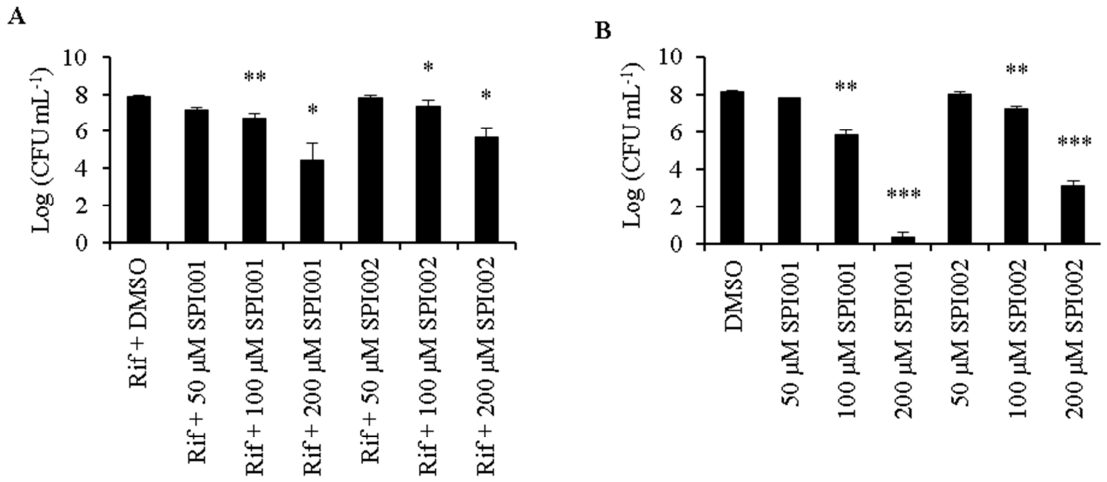


Figure 6.14: Effect of SPI001 and SPI002 on *S. aureus*. A. Anti-persistence and **B.** bactericidal effect was evaluated by treating stationary phase cultures for 5 hours with a combination of rifampicin (Rif) and compound or the compound alone, respectively. After treatment, cells were washed, diluted and plated on solid medium to determine the number of surviving cells. Data points represent the average of three independent repeats, error bars indicate SEM. *P < 0.1, **P < 0.05 and ***P <0.005.

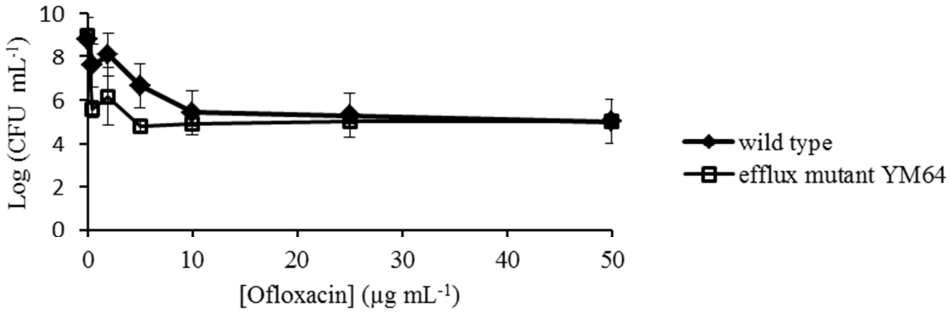


Figure 6.15: Optimization of persistence assay for the efflux mutant YM64 with ofloxacin. Stationary phase cells of wild type and efflux mutant YM64 were treated for 5 hours with increasing concentrations of ofloxacin. After treatment, cells were washed, diluted and plated on solid medium. Data points represent the mean of three independent repeats, error bars indicate SEM.

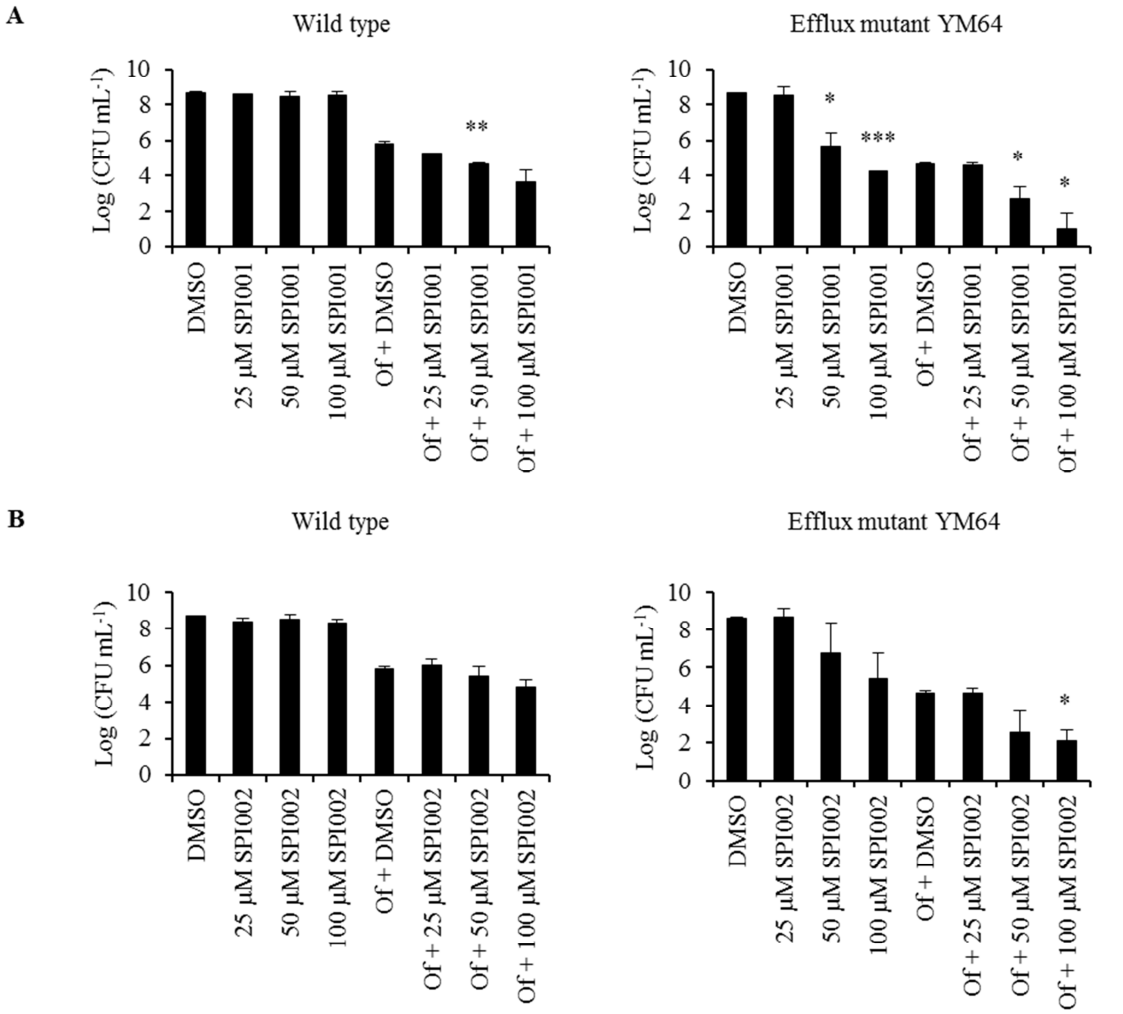


Figure 6.16 : Effect of SPI001 and SPI002 on efflux mutant. Anti-persistence and the bactericidal effect of A. SPI001 and **B.** SPI002 was evaluated on the wild-type and efflux mutant strain. Stationary phase cells were treated for 5 hours with the combination of ofloxacin and compound or compound alone. After treatment, cells were washed, diluted and plated on solid medium to determine the number of viable cells. Data points correspond to the average of three independent repeats, error bars represent the SEM. *P < 0.05, **P < 0.005 and ***P < 0.0005.

6.4 Discussion

In this Chapter, we described the identification of small molecules that significantly reduce the persister fraction in combination with the fluoroquinolone ofloxacin compared to ofloxacin monotreatment. Based on SAR analysis, the most promising anti-persister compounds, named SPI001 and SPI002, were selected for further characterization. We showed that these molecules are capable of reducing the persister fraction of *P. aeruginosa* in

combination with two additional clinically used antibiotic classes, and, in addition, have the ability to reduce the persister fraction of other relevant Gram-negative and Gram-positive pathogens.

6.4.1 Identification and selection of anti-persister molecules

For the initial selection of promising molecules, the effect was assessed by taking advantage of the observed correlation between the number of surviving cells and the lag phase after dilution of the surviving cells into fresh growth medium. Based on this screening method, we identified 15 different molecules that were able to reduce the persister fraction at least fourfold, corresponding to a hit rate of 0.063 %. Independent confirmation experiments by viable cell counts further reduced this rate to 0.021 % which is low in comparison with the only other similar screening reported in literature, for which a hit rate of 0.12 % was reported (Kim *et al.*, 2011). In this reported screening, chemicals that lowered the persister fraction of exponential growing *E. coli* cells in combination with ampicillin without acting growth inhibitory were identified. This difference can be explained by several factors. First of all, it is reported that the outer membrane of *P. aeruginosa* is approximately 100-fold less permeable compared to *E. coli* (Yoshimura & Nikaido, 1982), hereby reducing the number of molecules able to enter the bacterial cell to exert their action. This is supported by the observation that, when evaluating a collection of previously approved drugs to detect potentiation of the tetracycline antibiotic minocycline, the number of molecules showing activity against *E. coli* was seven times higher compared to the ones active against *P. aeruginosa* (Ejim *et al.*, 2011). Secondly, the use of a different start culture, mid-exponential versus stationary phase cultures in our set-up respectively, may explain our lower hit rate. For instance, it has been described that persisters generated during exponential phase are slow-growing cells rather than non-growing cells (Balaban *et al.*, 2004) making them more susceptible than non-growing cells. Furthermore, we previously showed that persisters formed during exponential phase conditions are more prone to resuscitation by which they regain susceptibility against antibacterial agents (Kristine Stepanyan, unpublished data). Lastly, the likely difference in composition of the used small-molecule collections probably affects the number of molecules identified.

Of the 15 selected compounds, only five were retained after analysis by viable cell counting. Additionally, after dose-response analysis, no correlation between the screening method and the viable cell counting experiments was detected for some of the compounds. The most striking example is provided by CIM056268, for which no effect on the persister fraction at all was detected by viable cell evaluation. Since treatment of the cells is carried out in exactly the same way, the difference in result is caused by factors inherently embedded in the evaluation method used to estimate the effect on persistence. Possibly, treatment with these compounds inhibit specific cellular pathways delaying regrowth after dilution of the surviving population in fresh medium. This results in the observation of a longer lag phase, without actually affecting the number of surviving cells. This hypothesis can be examined by treating isolated ofloxacin-tolerant persisters with compound or DMSO, dilute cells into fresh growth medium and monitor regrowth with viable cell counting.

In addition, molecules that in combination with ofloxacin result in the survival of more cells compared to monotreatment with ofloxacin, thus resulting in a shorter lag phase after dilution of treated cells into fresh growth medium, were identified during our screening (Appendix B, Table B.1). Possibly, these compounds increase the number of viable cells by interfering with the bactericidal action of ofloxacin. Alternatively, treatment with these molecules may stimulate regrowth of ofloxacin-tolerant cells upon encountering fresh growth medium. Unraveling the mode of action of such a persister regrowth-stimulating molecule may provide insight into signals or mechanisms implicated in the reversion of the dormant state of persisters to antibiotic-susceptible cells.

6.4.2 Combination with other mechanistically distinct antibiotics

Our screening was carried out by combining compounds with the fluoroquinolone antibiotic ofloxacin. It is suggested in literature that the multi-drug tolerant persister population is heterogeneous, composed of several subpopulations of persister cells, each represented by their own mechanisms of tolerance (Allison *et al.*, 2011b). As a consequence, individual persister cells can differ in their tolerance profile, as supported by the observed difference in persister fraction upon treatment of a population with distinct classes of antibiotics (Spoering & Lewis, 2001; Keren *et al.*, 2004a; Goneau *et al.*, 2014). Approaches have been reported in which the susceptibility of persisters cells to one specific antibiotic class can be increased,

hereby targeting a specific subpopulation of persister cells (Lu & Collins, 2009; Allison *et al.*, 2011a; Barraud *et al.*, 2013). In contrast, some molecules have been described which alter tolerance of persister cells to multiple antibiotic classes (Kim *et al.*, 2011; Pan *et al.*, 2012).

Our results show that SPI001 and SPI002 are able to reduce the persister fraction in combination with two additional mechanistically different classes of antibiotics. Combination of our two selected anti-persister molecules with the aminoglycoside amikacin, which acts by inhibiting translation, significantly reduced the persister fraction. The combination with SPI001 even resulted in total elimination of the bacterial population after a 5 h treatment. Similar results were obtained when combining the anti-persister molecules with the cephalosporin antibiotic ceftazidime, acting on cell wall biogenesis. These results indicate that our molecules are able to target multiple subpopulations of persisters, making them a promising starting point for the development of a future anti-persister therapy.

Based on these results, the identified anti-persister molecules may act by reverting antibiotic tolerance of the persister cells after which they are killed by the antibiotic present in the growth medium or, alternatively, by killing the persister cells itself. In literature, examples of both strategies were reported. For instance, the molecule 3-[4-(4-methoxyphenyl) piperazin-1-yl] piperidin-4-yl biphenyl-4-carboxylate (C10) wakes up persister cells (Kim *et al.*, 2011), while the synthetic quorum-sensing inhibitor (Z)-4-bromo-5-(bromomethylene)-3-methylfuran-2(5H)-one (BF8) has been described to revert multidrug tolerance in *P. aeruginosa* and *E. coli* strains without reverting persisters to growing cells (Pan *et al.*, 2012; Pan *et al.*, 2013a; Pan *et al.*, 2013b). On the other hand, the acyldepsipeptide antibiotic ADEP4 (Conlon *et al.*, 2013) and the anti-pseudomonal artilysin Art-175 (Briers *et al.*, 2014a) were shown to directly kill persister cells.

In addition, SPI001 and SPI002, although not bactericidal on *P. aeruginosa* stationary phase cultures, affect viability of growing cells (Figure 6.11). It was reported that *E. coli* exponential phase cells, stationary phase cells and persister cells represent three different physiological states (Shah *et al.*, 2006). Therefore, it is possible that the selected anti-persister molecules interfere with a process only important in exponential and persister state or that stationary phase cells are better protected against the cidal treatment. Non-growing stationary phase cells might be less susceptible than growing cells towards the action of our anti-persister molecules and, in addition, unlike metabolically quiescent persister cells, are able to

restore the damage caused by the treatment more quickly. Alternatively, the anti-persister molecules may induce non-specific side effects which are only lethal to growing cells.

6.4.3 Effect on other bacterial species

Although some processes involved in persister formation are shared among different bacterial species, such as for example the stringent and oxidative stress response, bacterium-specific differences are reported (see Chapter 2). An example is provided by the phenomenon of quorum sensing (QS), a process that regulates gene expression based on population density through the production and detection of signaling molecules (Waters & Bassler, 2005). This process was shown to be involved in the formation of *P. aeruginosa* persisters (Kayama *et al.*, 2009; Moker *et al.*, 2010; Que *et al.*, 2013), while it does not seem to play a role in the formation of persisters in *E. coli* (Lewis, 2007).

Therefore, we investigated the effect of SPI001 and SPI002 in combination with a conventional antibiotic against other clinically relevant bacterial species. SPI001 and SPI002 both reduced the persister fraction of the Gram-negatives *E. coli* and *S.* Typhimurium and of the Gram-positive *S. aureus*. Based on these results, we conclude that SPI001 and SPI002 act on a process involved in persister formation or interfere with a process essential for viability or maintenance of the antibiotic tolerant state which is shared among the tested bacteria.

While SPI001 and SPI002 did not affect viability of *P. aeruginosa* stationary phase cultures, these anti-persister molecules act bactericidal on stationary phase cultures of *E. coli*, *S.* Typhimurium and *S. aureus*. Possibly, the high intrinsic resistance of *P. aeruginosa* compared to other Gram-negatives (Breidenstein *et al.*, 2011) prevents the bactericidal action of SPI001 and SPI002. This is supported by the observation that stationary phase cultures of a *P. aeruginosa* efflux mutant are indeed susceptible to the bactericidal action of the anti-persister molecules (Figure 6.16).

6.4.4 Conclusion

We identified small-molecules belonging to three chemically distinct classes that, when combined with a conventional antibiotic, significantly reduce the persister fraction of *P.*

aeruginosa. Based on SAR analysis, the two most promising compounds were selected for further characterization. The anti-persister effect is not restricted to a specific antibiotic class nor to a specific bacterial species. These data strongly support the potential of these molecules to serve as a starting point for the development of an anti-persister therapy. A logical next step in the functional analysis of these anti-persister molecules would consist in unraveling their mode of action (see next Chapter).

Chapter 7

Detailed examination of biological activity and mode of action of the anti-persister molecule SPI001[14]

7.1 Introduction

Pseudomonas aeruginosa is one of the dominant bacteria present in the lungs of cystic fibrosis (CF) patients (Zhao *et al.*, 2012). Often, these infections become chronic thereby continuously damaging the lung which ultimately leads to respiratory failure (Folkesson *et al.*, 2012). When comparing clonal *P. aeruginosa* isolates from early and late stages of chronic infections, a 100-fold increase in persister cells was observed, which provides evidence for a causal relationship between the presence of persister cells and the recalcitrant nature of infections in the CF lung (Mulcahy *et al.*, 2010). In addition, persisters provide a reservoir of viable cells within the host, increasing the probability of acquiring additional resistance mechanisms by horizontal gene transfer or mutation, thereby contributing to the development of multidrug resistant strains (Cohen *et al.*, 2013). Therefore, targeting persisters will not only greatly help in clearing infections but also in preventing development of resistance.

Currently no molecular targets are available for rational development of an anti-persister therapy. Therefore, we used a top-down approach to identify small molecules that specifically act on persister cells. A medium-throughput screening was carried out for compounds that, in combination with the clinically used antibiotic ofloxacin, reduce the persister fraction of *P. aeruginosa*. We showed that the identified compounds can eliminate an entire bacterial culture in combination with mechanistically distinct antibiotic classes. Furthermore, they also exhibited activity against other Gram-negative pathogens such as *Escherichia coli* and S*almonella.* Typhimurium and against the Gram-positive bacterium *Staphylococcus aureus*

[14] Analysis of transcriptome data described in this Chapter was performed by Dr. Carolina Fierro (KU Leuven)

(Chapter 6). In this Chapter, one of those compounds, named SPI001, is examined in more detail. Here, we evaluate the effect of SPI001 on several *P. aeruginosa* clinical isolates and examine its combination spectrum in more detail. Furthermore, its mode of action is studied both at the cellular and genetic level.

7.2 Experimental procedures

Bacterial strains, media and growth conditions. Strains were grown in 1:20 Trypticase Soy Broth (TSB) at 37 °C. For solidified medium, TSB was supplemented with 1.5 % agar. The following antibiotics and antiseptics were used: ofloxacin, amikacin, ceftazidime, polymyxin B, hexachlorophene, acriflavine hydrochloride and ferric chloride. Concentrations are indicated in the text. All chemicals were purchased from Sigma unless indicated otherwise. Strains are listed in Table 7.1, clinical *P. aeruginosa* isolates were kindly provided by Prof. Françoise van Bambeke (Université Catholique de Louvain).

Minimal inhibitory concentration (MIC) determination. MIC values were determined as described before (see Chapter 4) using 1:20 TSB as a growth medium. The lowest antibiotic concentration resulting in the absence of bacterial growth was considered as the MIC.

Persistence assay. Determination of the number of persisters was carried out as described in Chapter 6.

Statistical analysis. Statistical analysis was carried out as described in Chapter 6.

Treatment of isolated persister cells. Persister cells were isolated as described previously with minor modifications (Pan *et al.*, 2012). Stationary phase cells were treated for 5 hours with ofloxacin (10 μg mL^{-1}). These conditions were previously optimized as higher ofloxacin concentrations or increased incubation times do not lead to a further reduction of the number of surviving cells. The surviving persisters were washed twice with a 0.85 % NaCl solution (4750 rpm, 15 minutes, 4 °C). These isolated persister cells were used for treatment as described in the text. After treatment, the sample was washed twice with 0.85 % NaCl (6000 rpm, 10 minutes, 4 °C) and divided into three parts. The first aliquot was diluted and plated out to determine the number of surviving colony forming units (CFU). The second and third aliquot were treated for 5 hours with water and ofloxacin respectively to determine the

number of persister cells that regained sensitivity to ofloxacin. Next, cells were washed, diluted and plated on solid medium in order to quantify the number of viable cells.

Table 7.1: strains used in this Chapter

Strain	Description[a]	Source or reference
P. aeruginosa PA14	Wild type, Km^R	Lee *et al.* (2006)
P. aeruginosa PAO1	Wild type	Dieter Haas (ETH)
P. aeruginosa YM64	*mexAB-oprM::FRT*, *mexXY::FRT*, *mexCD-oprJ::FRT*, *mexEF-oprN::FRT*	Morita *et al.* (2001)
P. aeruginosa BE136	Clinical isolate bronchus	Pirnay *et al.*(2002)
P. aeruginosa PA1255	Clinical isolate CF lung	Françoise van Bambeke (UCL)
P. aeruginosa AA249	Clinical isolate burn wound	Pirnay *et al.* (2002)
P. aeruginosa BU004	Clinical isolate throat	Pirnay *et al.* (2002)
P. aeruginosa BR642	Clinical isolate bed pan	Pirnay *et al.* (2002)
P. aeruginosa PA1256	Clinical isolate CF lung	Françoise van Bambeke (UCL)
E. coli SX43Δ*mutS*	K-12 MG1655 *lacZ*::*tsr-venus*; *mutS*::Km^R	Toon Swings (KU Leuven)

Synthesis of small unilamellar vesicles (SUV). SUV composed of 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE)/ 1,2-dioleoyl-sn-glycero-3-phospho-(1'-rac -glycerol) (DOPG) and 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC)/cholesterol representing bacterial and eukaryotic cell membranes respectively were prepared. All lipids were purchased from Avanti-Polar Lipids, Inc. Lipids, DOPE/DOPG (4:1 molar ratio) or DOPC/cholesterol (1:1 molar ratio) were mixed in dichloromethane, after which the solvent was removed by drying the sample using a flow of nitrogen gas followed by a three hour incubation in a excicator. Next, the lipid micelles were hydrated by adding a 50 mM carboxyfluorescein solution (50 mM carboxyfluorescein in HEPES/NaOH buffer, pH 7.4-7.5) and stirred with a glass stirrer. The lipid suspension was incubated at room temperature for 15 hours in a dark environment. The following day, the suspension was sonicated at 0 °C for 45 minutes. The SUV and free carboxyfluorescein were separated by exclusion chromatography

on a Sephadex G-75 column (diameter 2 cm, height 60 cm), using a 5 mM HEPES solution (pH 7.4) as eluent.

Determination of SUV concentration. The SUV concentration was determined by measuring the total fraction of inorganic phosphate. First, the organic phosphate was completely ashed as follows: a drop of magnesium nitrate solution was added to a SUV aliquot after which it was taken to dryness by heating the sample in a flame. Next, the total amount of inorganic phosphate was determined as described by Ames (1966). Briefly, a 10 % ascorbic acid: 0.42 % ammonium molybdate solution (1:6 v/v) was added to the sample and incubated for 20 minutes at 45 °C after which absorbance was measured at 820 nm. Ammonium molybdate was solubilized in 1N H_2SO_4 The readings were proportional to phosphate concentrations to an optical density of at least 1.8, 0.01 µmol of inorganic phosphate results in an absorbancy of 0.260.

Leakage assay. The phospholipid concentration is kept constant throughout the experiments ([DOPE+DOPG] or [DOPC+cholesterol] = 25 µM). SUV were treated with increasing compound concentrations, keeping the final dimethyl sulfoxide (DMSO) concentration at 1 % (v:v). After rapid and vigorous stirring, the time-course of fluorescence change corresponding to carboxyfluorescein release was recorded at 515 nm, with λ_{exc} 490 nm, for 15 minutes. The percentage of released carboxyfluorescein at time *t* was determined by:

$$\% \text{ carboxyfluorescein} = (F_t - F_0)/(F_T - F_0) \times 100\ \%$$

With: F_0 = intensity of fluorescence of the liposomes in absence of peptide; F_t = intensity of fluorescence at time *t* in the presence of peptide; F_T = intensity of fluorescence after liposome destruction by adding 50 µL 10 % Triton X-100 in water.

Sample preparation and RNA sequencing. Overnight cultures were diluted 100-fold into fresh 1:20 TSB medium and grown until late-exponential phase (OD_{595} = 0.2). Next, cells were treated for 15 minutes with 50 µM SPI001, 50 µM of the inactive analogue CIM056276 (see Figure 6.6), further referred to as SPI006, or 1 % DMSO. RNA sampling, isolation and assessment of RNA integrity, quantity and purity was carried out as described before (see Chapter 4). For each strain, RNA isolation was done in triplicate. To deplete ribosomal RNA sequences, the samples were treated with the Ribo-Zero™ rRNA Removal Kit for Gram-

negative bacteria (Epicentre) according to the manufacturer's instructions. Preparation of the library followed by RNA sequencing was performed by GeneCore, EMBL, Heidelberg, Germany.

Transcriptome data analysis. Raw RNA-Seq data were evaluated with FastQC for quality control. Only one of the nine samples (cells treated with SPI006) showed a bias in its base content along the whole read length. Multivariate analysis performed with the mpm R package (Wouters *et al.*, 2003) and hierarchical clustering of samples indicated that the sample largely differed from the rest and it was removed from subsequent analyses. Genomic alignments of reads were performed with Bowtie2 using *P. aeruginosa* UCBPP-PA14 genome as reference (NC_008463.1). Raw counts for gene expression were obtained with HTSeq-count (no strain-specific option and union mode to count for overlapping features). Differential expression between treated and control samples was done with DESeq2 (Anders & Huber, 2010; Love *et al.*, 2014) using a False Discovery Rate threshold of 5%. The list of differentially expressed genes was further refined by keeping log2 fold-changes above 1. For further analysis of genes affected upon treatment with the anti-persister molecule SPI001, genes differentially expressed under the inactive compound treatment were removed. Gene expression values for plotting were obtained using the regularized log-transformation in the DESeq2 package. Gene expression values were mean centered and variance rescaled before visualization with heatmaps. Functional annotation of results was performed using KEGG pathways and KEGG modules. PseudoCAP functional classes and Gene Ontology annotation were obtained from www.pseudomonas.com (Winsor *et al.*, 2011). Since the functional annotation for the UCBPP-PA14 strain is more limited than for the PAO1 strain, we finally used the PAO1 gene annotation and transferred it to UCBPP-PA14 strain using orthology relations from the OMA browser (Altenhoff *et al.*, 2011).

Network analysis. *Pseudomonas* functional interactions were obtained from STRING database (Jensen *et al.*, 2009). Only gene-gene interactions with a minimum reliability score of 0.8 were retained. A deregulated network was extracted by selecting interactions in which both genes are differentially expressed in the presence of the active compound. Network visualization was done with Cytoscape (Smoot *et al.*, 2011).

7.3 Results

7.3.1 Effect of SPI001 on *Pseudomonas aeruginosa* clinical isolates

Until now, the anti-persister effect of SPI001 had only been tested on the *P. aeruginosa* lab strain PA14, a human clinical isolate (Rahme *et al.*, 1995). To assess if SPI001 is also active against other clinical isolates, previously exposed to different *in vivo* conditions, we obtained *P. aeruginosa* isolates from different sampling sites (Table 7.2).

Table 7.2: Clinical *P. aeruginosa* strains and their characteristics. Source and collection information is given with information regarding serotype (Sero), siderophore type (pyoverdine type, PVD), presence of *exoS*/*exoU* and *oprD* allele code based on data published by Pirnay *et al.* (2009).

Strain	Source	Treatment concentration ($\mu g\ mL^{-1}$)			Resistance*	Sero	PVD	exoS/ exoU	*oprD***
		Am	Of	Cf					
BE136	bronchus	100	100	250	/	3	IIa	*exoU*	A110
PA1255	CF lung	100	10	250	/	/	/	/	/
AA249	burn wound	500	100	250	Am, Of, Cf, IPM, MEM, ATM & Cp	NT	I	*exoS*	B107 (DOM)
BU004	throat	100	100	250	/	NT	I	*exoS*	C106
BR642	bed pan	100	100	250	/	1	I	*exoU*	A103
PA1256	CF lung	100	10	250	/	/	/	/	/

* Based on data of Kristine Stepanyan and Pirnay *et al.* (2002) which were compared to EUCAST values[15]. Am, amikacin; ATM, aztreonam; Cf, ceftazidime; Cp, ciprofloxacin; IPM, imipenem; MEM, meropenem; Of,ofloxacin

**DOM, defective *oprD* mutation

Stationary phase cells were treated for 5 hours with an antibiotic or a combination of antibiotic and 200 µM SPI001. Based on MIC-testing carried out in our research group (data

[15] http://www.eucast.org/clinical_breakpoints/

of Kristine Stepanyan, not shown), antibiotic concentrations that only allowed survival of persister cells were determined. After treatment, cells were washed, diluted and plated on solid medium. The lab strains PA14 and PAO1 were included as reference. The results are shown in Figure 7.1. The majority of isolates tested was extremely susceptible to the combination of ofloxacin and SPI001 resulting in complete eradication of the bacterial population. Treatment of PA1255 and AA249 resulted in a ~15 and ~90 times lower persister fraction respectively compared to the ofloxacin monotreatment. Combination of SPI001 with the cephasloporin ceftazidime decreased the persister fraction at least ~150-fold of all clinical isolates tested. The best result was obtained with the CF lung isolate PA1256, where combination of ceftazidime with SPI001 resulted in a ~7900 times lower fraction compared to treatment with only the antibiotic. Finally, combining SPI001 with the aminoglycoside amikacin also significantly decreased the persister fraction compared to monotreatment. However, for clinical isolates AA249 and BU004 and the lab strain PAO1, no persister fraction was observed after treatment with the aminoglycoside amikacin.

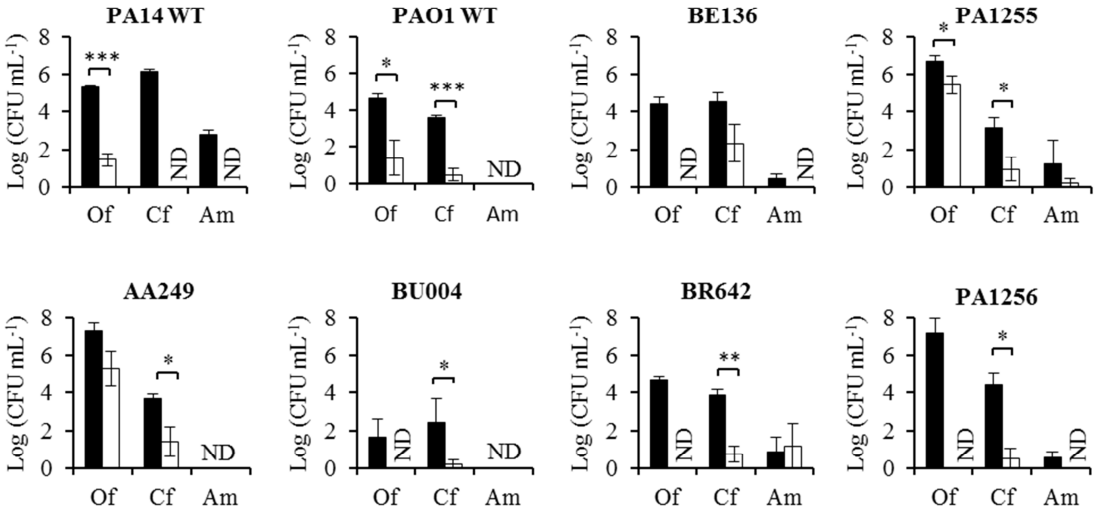


Figure 7.1: Effect of SPI001 in combination with clinically used antibiotics on *P. aeruginosa* clinical isolates. Stationary phase cells were treated for 5 hours with ofloxacin (Of) or amikacin (Am) alone or combined with SPI001. After treatment, cells were washed, diluted and plated on solid medium to determine the surviving cells. To test the combination of ceftazidime (Cf) with SPI001, dividing cells were treated for 5 hours. Data points represent the mean of three independent repeats, error bars represent standard error of the mean (SEM). Black and white bars represent the result after monotreatment and combination treatment (the indicated antibiotic and SPI001) respectively. *P < 0.05, **P < 0.005 and *** P < 0.0005. ND, not detectable.

Aminoglycosides are an important class of antibiotics frequently used as treatment of *P. aeruginosa* infections. In order to evaluate the effect of SPI001 in combination with other aminoglycosides on these strains, the experimental conditions were optimized using gentamicin and tobramycin (data not shown). Unfortunately, it was not possible to obtain a stable persister fraction in this experimental setup.

7.3.2 Combination spectrum of SPI001

To further characterize the combination spectrum of SPI001, Phenotype MicroArrays (Biolog) were used. These arrays contain 240 different compounds, supplied in four different concentrations that target specific cellular pathways[16]. To avoid confusion, these compounds will be further referred to as 'Biolog compounds'. The goal was to identify Biolog compounds that, in combination with SPI001, reduce the number of surviving cells compared to treatment with the Biolog compound alone. To lower the amount of SPI001 needed for the initial screening of these Phenotype MicroArrays, the *P. aeruginosa* efflux mutant YM64 was used, which is more sensitive towards SPI001 (see Section 6.3.4.3). Subsequent confirmatory experiments were carried out using the PA14 wild-type strain.

The screening method is based on the correlation between the number of surviving cells and the duration of the lag phase (see Section 6.3.1). Essentially, the lower the numbers of surviving cells, the longer it takes for this remaining bacterial population to yield visible growth after dilution into fresh growth medium. For practical reasons, treatment time was prolonged and stationary phase cells were treated for 24 hours. Regrowth was monitored by measuring the optical density (595 nm) at successive time points by using a microplate reader (BioTek® Synergy HT Multi-Mode Microplate Reader).

7.3.2.1 Optimization of screening conditions

To determine the dilution factor of treated cells, a first experiment was carried out in which stationary phase cultures were treated with increasing amounts of ofloxacin (0-5 µg mL^{-1})(MIC value of ofloxacin on YM64 = 0.04 µg mL^{-1}). After treatment, cells were diluted 100-

[16] www.biolog.com/products-static/phenotype_microbial_cells_use.php

or 1000-fold into fresh medium and regrowth was monitored. Lower dilutions were not taken into consideration to avoid effects of residual ofloxacin on regrowth. Since differences in regrowth were more obvious after a 100-fold dilution of the treated cells, this factor was chosen for further experiments (Figure 7.2).

Treatment of stationary YM64 cultures with 5 µg mL^{-1} ofloxacin prevented regrowth of the 100 and 1000-fold diluted surviving population. A 100-fold dilution of the antibiotic leaves a residual concentration of ofloxacin above the MIC value, which possibly explains the lack of regrowth of the surviving population. However, a 1000-fold dilution brings the residual ofloxacin concentration back to a level below the determined MIC value. Therefore, it seems that a 24 h treatment of stationary phase cells with 5 µg mL^{-1} ofloxacin allows survival of a very low number of cells of which regrowth cannot be detected within 28 hours. This is in disagreement with results obtained after a five hour treatment of YM64 stationary phase cells (Section 6.3.4.3). Here, addition of 5 µg mL^{-1} ofloxacin or higher leaves a stable persister fraction of approximately 0.01 % which corresponds to 1 x 10^5 cells mL^{-1}.

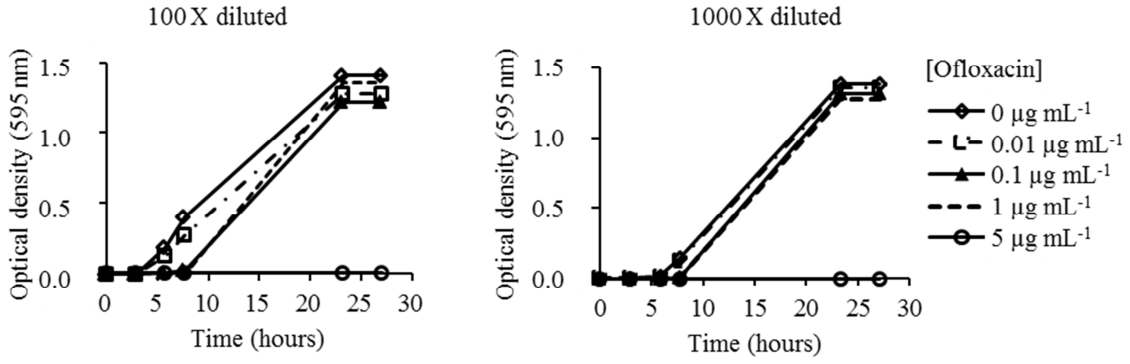


Figure 7.2: Optimization of dilution factor after treatment. Stationary phase cells were treated for 24 hours with increasing concentrations of ofloxacin (0 – 5 µg mL^{-1}, as indicated by the different symbols). After treatment, cells were 100- or 1000-fold diluted into fresh growth medium and regrowth was monitored by measuring optical density (595 nm).

The concentration of antibacterial agent present in these Phenotype Microarrays is unknown. To determine the optimal concentration of SPI001 to identify combination effects, the following experiment was carried out. Stationary phase cells were treated with ofloxacin (0-5 µg mL^{-1}) and 0, 25, 50 or 100 µM of SPI001 (MIC value of SPI001 on YM64 = 50 µM) for

24 hours after which the cells were diluted 100-fold into fresh medium to monitor regrowth. The results are given in Figure 7.3. As expected, treatment with 5 µg mL^{-1} ofloxacin prevented regrowth within 28 hours. Combination with 25 µM SPI001 prevented regrowth when cells were simultaneously treated with 1 µg mL^{-1} ofloxacin. When 50 or 100 µM SPI001 was administered together with ofloxacin, regrowth was only detected for cells treated with SPI001 in combination with the lowest ofloxacin concentration tested. Based on these data, a concentration of 50 µM SPI001 was selected.

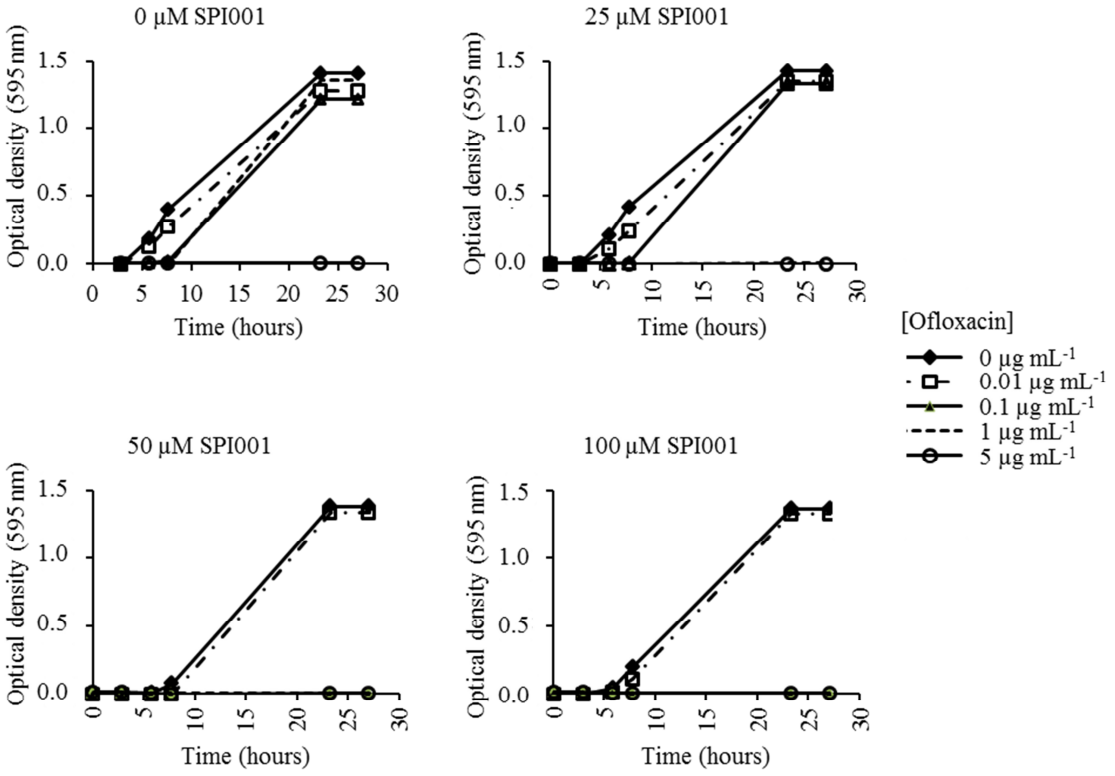


Figure 7.3: Optimization of SPI001 concentration. Stationary phase cells were treated for 24 hours with increasing concentrations of ofloxacin (as indicated by the symbols) combined with 0, 25, 50 or 100 µM SPI001. After treatment, cells were 100-fold diluted into fresh growth medium and regrowth was monitored by measuring optical density (595 nm).

7.3.2.2 Screening

Screening of the Phenotype Microarrays was carried out using conditions as described above, regrowth was monitored for 32 hours. Several Biolog compounds were identified that, in

combination with SPI001, reduced the number of surviving cells compared to the corresponding monotreatment. Clinically relevant Biolog compounds identified in our assay are listed in Table 7.3. Among these compounds, the fluoroquinolone ofloxacin and several aminoglycosides were detected. In the previous Chapter, we showed that SPI001 completely eliminates the bacterial culture in combination with these classes of antibiotics, hereby confirming our screening procedure.

Table 7.3: Biolog compounds identified during the screening

Class	Identified Biolog compounds
Antibiotics	
Fluoroquinolones	ofloxacin
Aminoglycosides	amikacin, neomycin, gentamicin, paromomycin, sisomicin, tobramycin
Polymyxins	polymyxin B
Tetracyclines	doxycycline
Peptidyl transferase	thiamphenicol
Aminonucleosides	puromycin
Antiseptics	ferric chloride, acriflavine, proflavine, hexachlorophene
Others	lidocaine, atropine, pridinol

For further experiments we selected two interesting classes of identified Biolog compounds. The first one is represented by the polymyxin antibiotics for which polymyxin B was identified during our experiment. This class of antibiotics acts by disrupting the outer and inner membrane of Gram-negative bacteria (as reviewed by Falagas *et al.* (2010)). As shown in Figure 7.4, combination of SPI001 with the highest concentration of polymyxin B available in the Phenotype Microarrays significantly affects regrowth of the treated bacterial culture.

Secondly, the identified Biolog compounds comprised several antiseptics (Figure 7.5). One of them is acriflavine, an antiseptic used for skin and mucous membranes (Kumar *et al.*, 2012).

Again, only combination of SPI001 with the highest concentration available in the Phenotype Microarrays significantly affects regrowth of the treated bacterial culture (Figure 7.4).

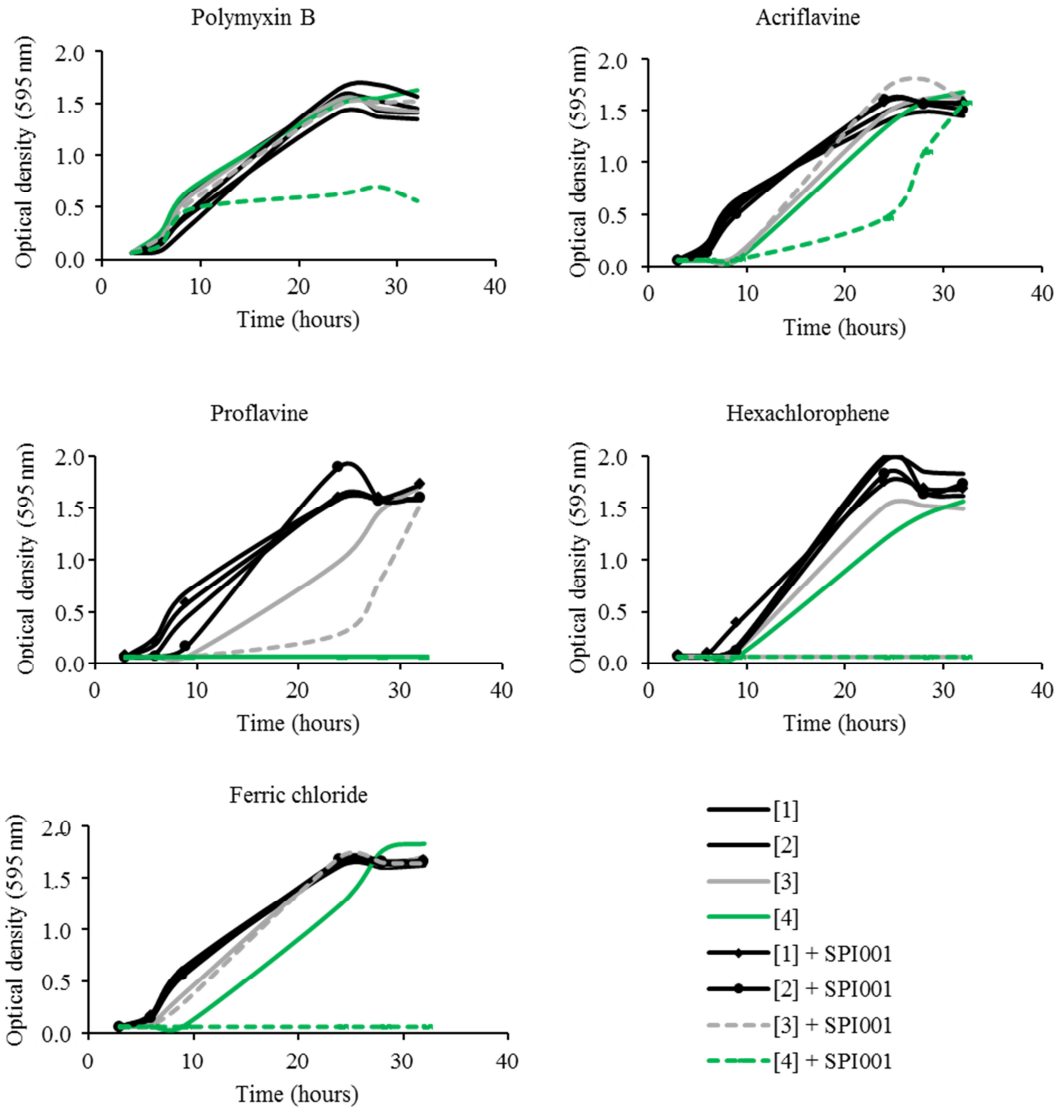


Figure 7.4: Effect of SPI001 in combination with selected Biolog compounds. Stationary phase cells were treated overnight with a combination of 50 µM SPI001 and four different concentrations of Biolog compound (designated by [1] to [4]). After treatment, cells were diluted 100-fold into fresh growth medium and growth was monitored by measuring optical density at fixed time points. As the effect is mainly visible upon treatment with the two highest concentrations ([3] and [4]) of the Biolog compound, these treatment regimens are shown in different colors.

Commercial preparations are often mixtures with proflavine, which was also identified during our screen. Here, the highest proflavine concentration present in the Phenotype Microarrays completely prevented regrowth when administered alone. However, a significant difference was detected between cells treated with the second highest concentration of proflavine alone or combined with SPI001 (Figure 7.4). For further experiments acriflavine hydrochloride, a mixture of acriflavine and proflavine, was purchased. A third antiseptic identified was hexachlorophene, which is widely used in drug and cosmetic products and is reported to be mainly active against Gram-positive bacteria. A combination of SPI001 with the two highest concentrations present in the Phenotype Microarrays completely eliminated regrowth (Figure 7.4). The last antiseptic identified is ferric chloride, which is used to protect wounds from infection. When the highest concentration of ferric chloride was combined with SPI001, no regrowth was observed (Figure 7.4). The result of the other Biolog compounds listed in Table 7.3 are available in Appendix B, Figure B.4.

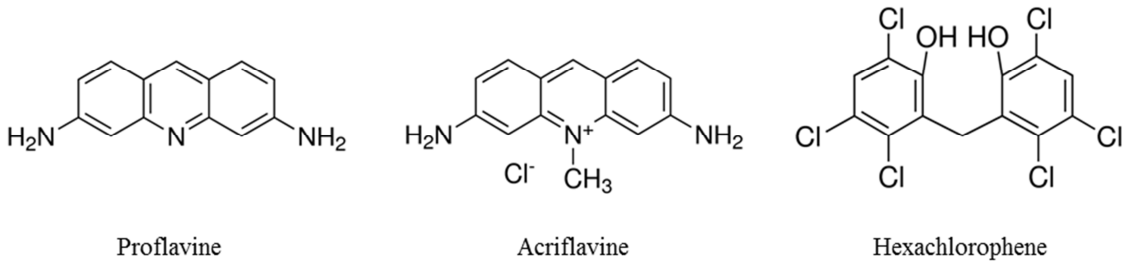


Figure 7.5: Chemical structures of the antiseptics proflavine, acriflavine and hexachlorophene.

7.3.2.3 Confirmation of the obtained results

For confirmatory experiments, we focused on the polymyxin antibiotic polymyxin B and the identified antiseptics ferric chloride, hexachlorophene and acriflavine hydrochloride. These experiments were carried out using the PA14 wild-type strain, the treatment time was reduced to 5 hours. In first instance, we evaluated the direct effect on persistence. MIC values of the Biolog compounds were determined (data not shown) and cells were treated with 10 X MIC combined with 200 µM SPI001 after which the number of viable cells was assessed. Based on earlier experiments carried out in our group, treating cells with 10 X MIC ensures survival of the antibiotic-insensitive persisters only. Treatment of stationary phase cells with a

combination of 6.25 µg mL^{-1} polymyxin B and 200 µM of SPI001 for 5 hours completely eliminated the bacterial population (Figure 7.6) further underlining the broad application range of SPI001. However, treatment with 10 X MIC of the three selected antiseptics did not yield a detectable persister fraction upon monotreatment (data not shown). Possibly, potentiating effects of SPI001 on lower concentrations of antiseptic were detected in the initial screening of the Phenotype Microarrays.

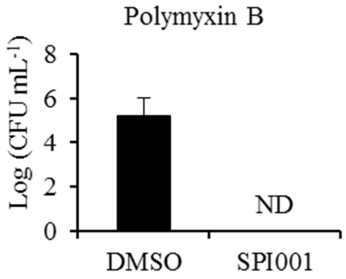


Figure 7.6: Effect of SPI001 in combination with polymyxin B. Stationary phase cells were treated for 5 hours with the combination polymyxin B (6.25 µg mL^{-1}) and DMSO (1 %) or with the combination polymyxin B (6.25 µg mL^{-1}) and 200 µM SPI001. After treatment, cells were washed, diluted and plated on solid medium to determine the number of viable cells. The experiment was independently repeated three times. Data points represent the mean, error bars indicate SEM. ND, not detectable.

To assess these possible potentiating effects, we tested different concentrations of the three antiseptics in combination with 200 µM SPI001 by monitoring regrowth of the treated stationary phase cultures after dilution in fresh medium. Results show that combination of SPI001 with MIC or sub-MIC concentrations of the antiseptics clearly delayed growth compared to monotreatment (Figure 7.7A). Next, plate counting experiments were carried out using sub-MIC (0.5 X MIC) concentrations of the respective antiseptics to confirm the observed effects. The results show that the bactericidal effect of acriflavine hydrochloride on stationary phase cells was potentiated upon addition of SPI001, hereby lowering the active concentration of antiseptic needed to obtain complete elimination of the bacterial population. However, the combination of hexachlorophene or ferric chloride with SPI001 did not result in a statistically significant difference in the number of surviving cells compared to monotreatment with the antiseptic (Figure 7.7B).

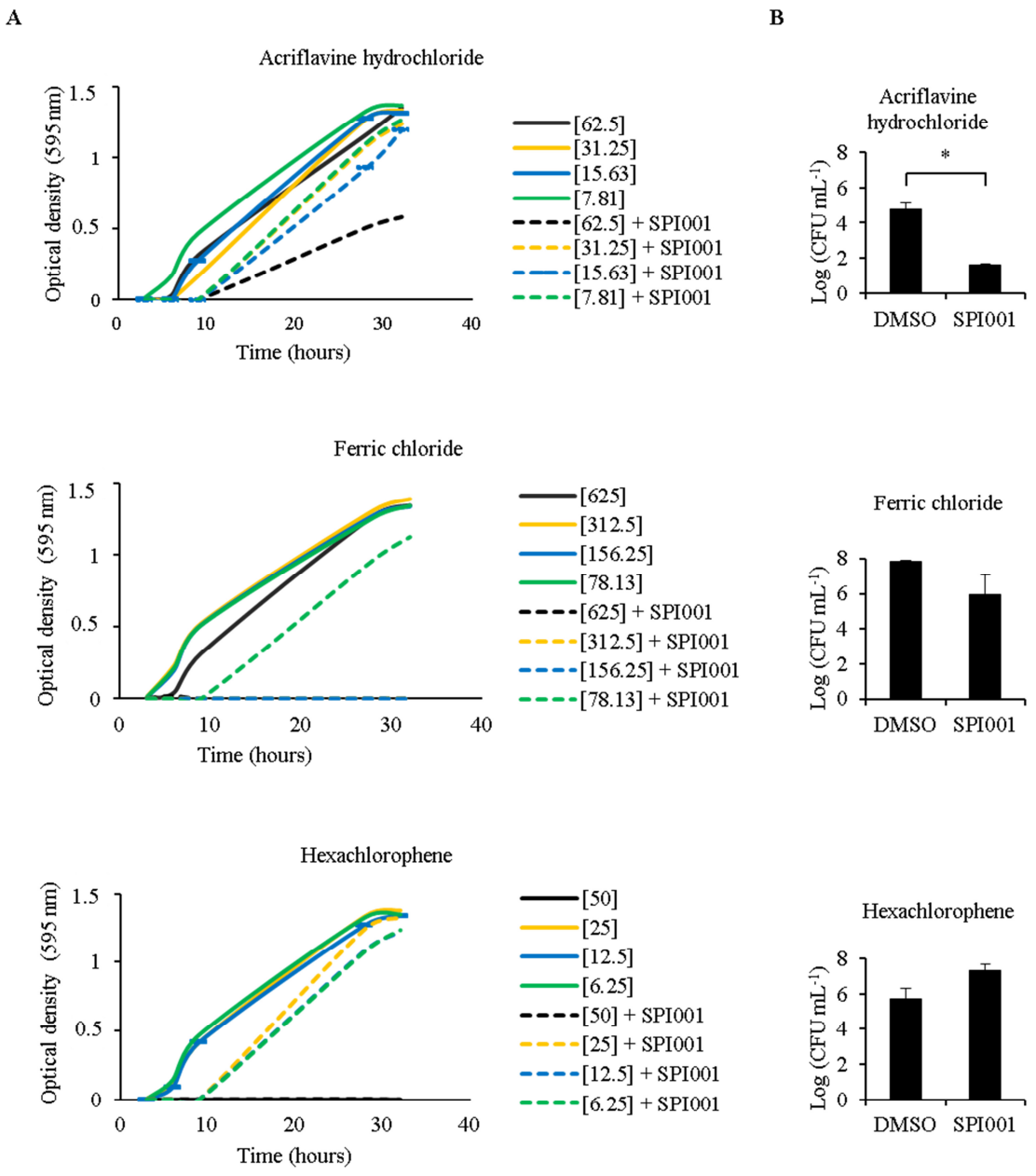


Figure 7.7: Effect of SPI001 in combination with antiseptics. A. Stationary phase cells of PA14 WT were treated overnight with a combination of Biolog compound (used concentrations (µg mL^{-1}) are indicated in the figure legend) and 200 µM SPI001. After treatment, cells were 100-fold diluted into fresh growth medium and regrowth was monitored by measuring optical density at fixed time points. **B.** Stationary phase cells were treated for 5 hours with a combination of antiseptic and 200 µM SPI001. After treatment, cells were washed, diluted and plated on solid medium to determine the number of viable cells. Each experiment was independently repeated three times. Data points represent the mean, error bars indicate SEM. For this experiment, 31.25 µg mL^{-1} acriflavine hydrochloride (0.5 X MIC), 312.5 µg mL^{-1} ferric chloride (0.5 X MIC) and 25 µg mL^{-1} hexachlorophene (0.5 X MIC) was used respectively. *P < 0.05.

7.3.3 SPI001 kills persister cells in the absence of antibiotics

To investigate whether SPI001 can also reduce the persister fraction when not administered simultaneously with ofloxacin, the following experiment was carried out. *P. aeruginosa* stationary phase cells were treated with ofloxacin (10 µg mL^{-1}) or water. After 5 or 24 hours, 200 µM SPI001 or 1 % DMSO was added and the number of CFU was monitored over time (Figure 7.8B and C). For comparison, stationary phase cells were treated for 72 hours with SPI001 or with the combination of ofloxacin and SPI001 (Figure 7.8A). For each experiment, complete eradication of the bacterial population was achieved within 24 hours after addition of SPI001. These results indicate that SPI001 can be administered separately from ofloxacin, hereby broadening treatment options. Although no bactericidal effect was detected when treating stationary phase cultures for 5 hours with SPI001 (see Chapter 6, Figure 6.6), this compound acts bactericidal upon increased treatment time (Figure 7.8).

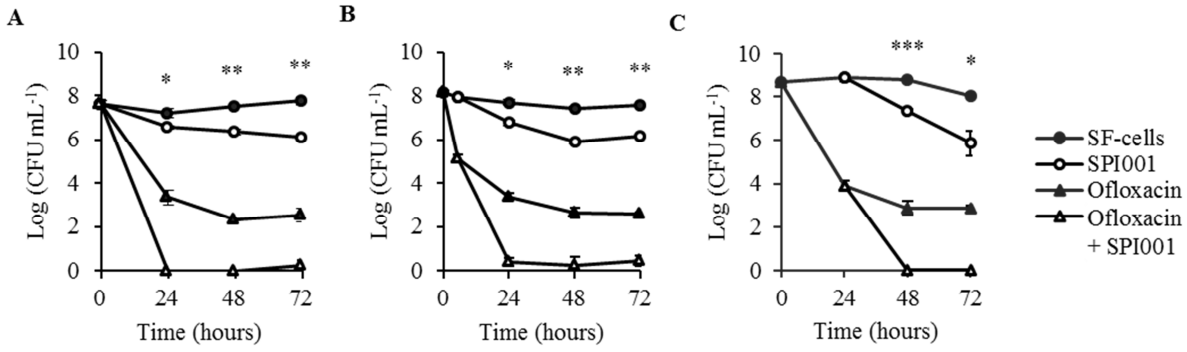


Figure 7.8: Effect of SPI001 on ofloxacin treated stationary cultures. A. Stationary phase cells were treated for 72 hours with SPI001 or the combination of ofloxacin and SPI001. **B.** stationary phase cells were first treated with ofloxacin or water for 5 or **C.** 24 hours after which SPI001 was added to the culture. During treatment the number of viable cells was determined at 24, 48 and 72 hours. Data points represent the average of three independent repeats, error bars indicate SEM. Statistical significance for the cidal effect of SPI001 on stationary phase cells on the different time points is displayed, with *P < 0.05, **P < 0.005 and ***P < 0.0005.

Based on these results, we can conclude that SPI001 may act in two different ways. One possibility is that SPI001 is capable of waking up the persister cells, hereby rendering them sensitive to the bactericidal action of ofloxacin present in the growth medium. Secondly, SPI001 could kill the persister cells directly. To distinguish between those two possibilities, persister cells were isolated and treated separately with 1 % DMSO, 10 µg mL^{-1} ofloxacin,

200 µM SPI001 or the combination ofloxacin and SPI001. After treatment, samples were divided into three aliquots. The first one was diluted and plated on solid medium to evaluate the effect of treatment. The second and third aliquot was treated for 5 hours with water and ofloxacin respectively. This allowed us to determine the fraction of cells that regained sensitivity towards ofloxacin. Treatment of isolated persisters with 200 µM SPI001 almost completely eliminated all cells, in contrast to the persister cells that had been subjected to an ofloxacin treatment. This result shows that SPI001 is capable of killing persister cells directly (Figure 7.9A).

The persister fraction slightly decreases upon treatment with ofloxacin compared to treatment with DMSO. Manipulation of the persister cells during the washing steps seems to sensitize a small fraction of persisters which are killed by ofloxacin present in the sample. This is supported by the results shown in Figure 7.9B. Therefore, it was evaluated whether treatment of persister cells in spent medium reduces the reversion of a small fraction persisters into ofloxacin-sensitive cells. However, again a small fraction of persister cells regained sensitivity towards ofloxacin (data not shown). Upon testing of the SPI001 treated persister cells for regained sensitivity towards ofloxacin, no cells were detected after control treatment with water. Likely, the low number of persisters cells surviving the treatment with SPI001 were washed away before plating out the ofloxacin and water treatment respectively.

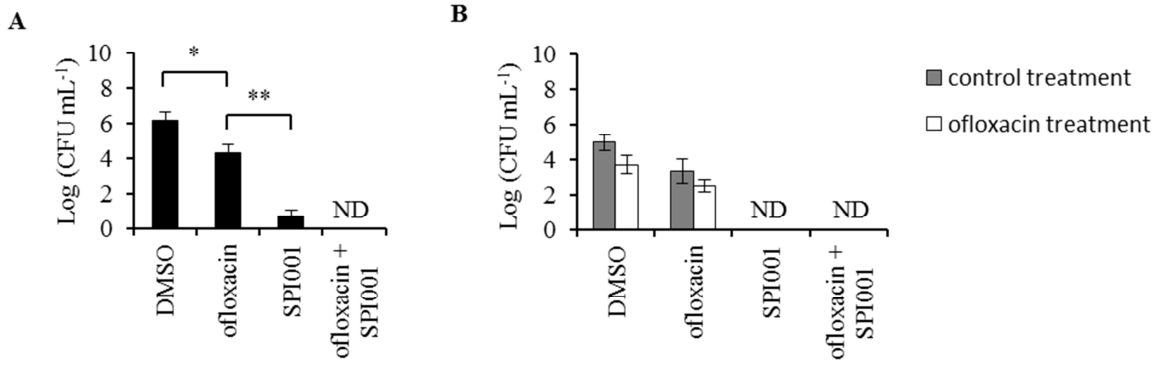


Figure 7.9: SPI001 kills persister cells. Persister cells were isolated and treated for 5 hours with 1 % DMSO, 10 µg mL^{-1} ofloxacin, 200 µM SPI001 or a combination of ofloxacin and SPI001. After treatment, the sample was divided into three parts and **A.** the first aliquot was diluted and plated on solid medium to determine the number of surviving persister cells. **B.** the second and third aliquots were treated for 5 hours with water and ofloxacin respectively. Data points correspond to the mean of three independent repeats, error bars represent SEM. *P < 0.05, **P < 0.005. ND, not detectable.

7.3.4 SPI001 induces membrane damage

Our results indicate that SPI001 is able to kill persister cells. Theoretically, *in vitro*, this can be achieved by interfering with processes essential to maintain viability (see Chapter 2). One such target, frequently proposed as suitable for killing dormant cells, is membrane integrity (Hurdle *et al.*, 2011). To investigate whether this might explain the observed anti-persister effect, we tested whether SPI001 is able to disrupt lipid bilayers that mimick the Gram-negative inner membrane. In addition, the membrane disrupting capacity of SPI001 was tested on SUV representing eukaryotic cell membranes. Additional analogues, representing small molecules with or without bactericidal and/or anti-persister activity, were included in this analysis as controls (Table 7.4).

Table 7.4: Overview of chemical analogues used in the carboxyfluorescein leakage assay. Persistence and bactericidal effect and MIC values on the PA14 wild type strain are shown

Analogue	Persistence effect	Bactericidal effect	MIC (µM)
SPI001 (CIM056271)	Yes	No	200
SPI003 (CIM056263)	No	No	>200
SPI004 (CIM056273)	No	Yes	200
SPI005 (CIM056307)	Yes	No	100

SUV were treated with increasing concentrations of compound for 15 minutes. Every minute, carboxyfluorescein leakage was monitored by measuring absorbance at 515 nm. For all samples, the maximum value was reached within a few minutes, therefore only the measurement at 15 minutes is shown. As a negative and positive control of the bacterial model, ofloxacin and polymyxin B were included (Figure 7.10). In line with our expectation, ofloxacin did not induce any leakage while polymyxin B caused leakage at relatively low concentrations. The maximum level of leakage was reached at a polymyxin B/lipid molar ratio of 0.11 which corresponds to a polymyxin B concentration of 2 µg mL^{-1} (Figure 7.10A). SPI003, an analogue displaying no bactericidal or anti-persistence effect, did not cause significant leakage in either the bacterial or eukaryotic system. Addition of SPI004, which displays a bactericidal but not a persister effect, caused leakage in both models. SPI005, an analogue which is capable of reducing the persister fraction in combination with ofloxacin

without displaying a bactericidal effect on stationary phase cells, induced significant leakage in the bacterial and eukaryotic system. However, leakage caused in the eukaryotic system is lower compared to the bacterial model. These data suggest that compounds affecting the persister fraction induce leakage in both system, but to a lesser extent in the eukaryotic model, while compounds that are bactericidal cause maximum damage in both systems. When testing the membrane disrupting capacity of SPI001, the lipid bilayer was disrupted in both *in vitro* models. The *in vivo* effect, however, seems to differ as no cytotoxicity was observed on human embryonic kidney 293 cells (LD_{50} > 200 µM) (Hugo Klaassen, personal communication).

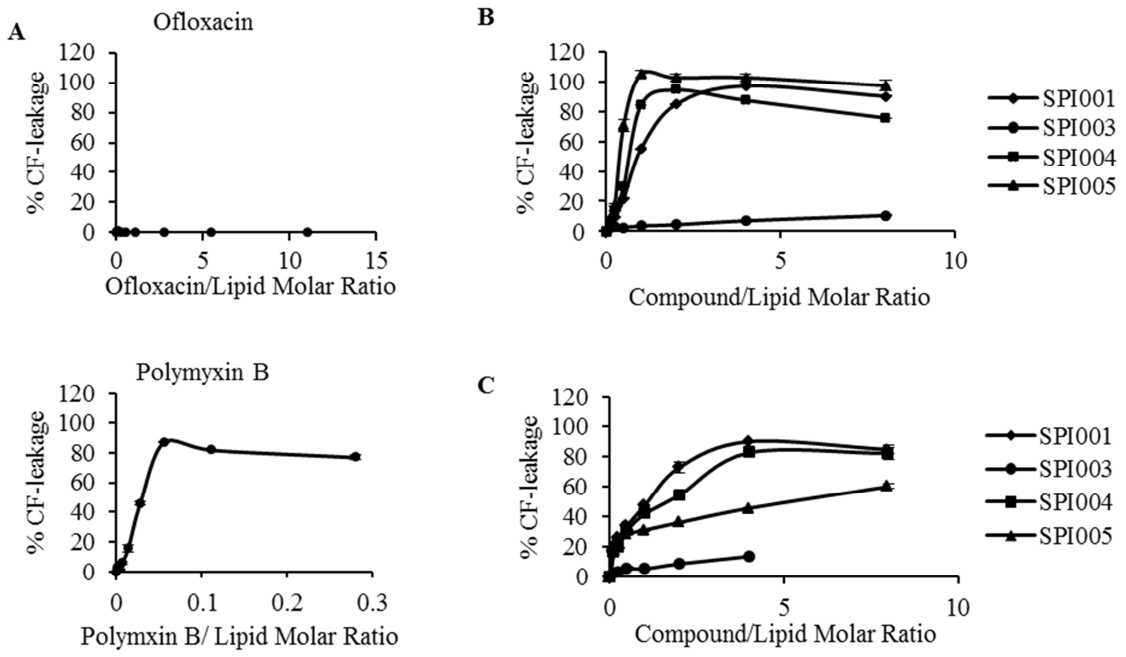


Figure 7.10: Carboxyfluorescein leakage assay. A. SUV representing the bacterial membrane were treated with increasing concentrations of ofloxacin (negative control) and polymyxin B (positive control). **B.** SUV representing bacterial or **C.** eukaryotic membranes were treated with SPI001 and three other chemical analogues. After 15 minutes, absorbance was measured. Data points represent the average of three independent repeats, error bars indicate SEM.

7.3.5 SPI001 spontaneous resistant mutants

Generation and analysis of spontaneous resistant mutants (SRM) provides insight in resistance mechanisms (Srinivas *et al.*, 2010; Mistry *et al.*, 2013). Here, we seek to isolate

SRM that are resistant to the growth inhibitory effect of SPI001. By additionally testing the SRM for their sensitivity towards the anti-persistence effect of SPI001, information is obtained on the causative correlation between the bactericidal and anti-persistence effect of SPI001 in *P. aeruginosa*.

P. aeruginosa YM64 cells (5 x 10^8, 1 x 10^9 and 5 x 10^9 cells as determined by plate counting) were plated on solid 1:20 TSB growth medium containing 4 and 5 times the MIC of SPI001 respectively. After three weeks of incubation at 37 °C, no colonies were detected.

To increase the probability of SRM development, an *E. coli mutS* mutant was used. This mutant was extensively characterized in our research group and has a 65-fold increased mutation rate compared to the corresponding wild-type strain (Toon Swings, personal communication). The MIC of SPI001 against this strain was determined (MIC value = 50 µM) and subsequently, 5 x 10^9 cells were plated on solid medium containing 2.5 or 4 times the MIC of SPI001. However, after three weeks of incubation at 37 °C, no SRM were formed.

7.3.6 Transcriptome analysis of cells exposed to SPI001

To assess the change in gene expression upon addition of SPI001 to growing cells, a transcriptome analysis was carried out. *P. aeruginosa* PA14 wildtype growing cells were treated with respectively SPI001, the inactive analogue SPI006 or the solvent DMSO for 15 minutes. A concentration of 50 µM (0.25 X MIC) was used since this causes no significant effect on viability of the cells, while a clear effect on growth was detected (Figure 7.11).

In total, 316 and 39 genes were differentially expressed (log2 fold change ≥ 1) upon treatment of growing cells with SPI001 and SPI006 respectively. 19 genes were differentially expressed upon treatment with both compounds and were removed from the list of genes differentially expressed upon treatment with SPI001. Because of the high number of genes affected upon treatment with SPI001, we based our analysis on the identification of functionally related processes targeted by different genes. For this end, a functional interaction network was constructed based on interactions in which both partners (genes) were differentially expressed in cells treated with SPI001. These sub-networks were annotated using KEGG pathways and KEGG modules, PseudoCAP functional classes and Gene Ontology annotation and are shown

in Figure 7.12. Interaction clusters that consisted of only two genes or that could not be classified into one or more functional categories were excluded for further analysis. The obtained network consisted of 88 genes and 404 interactions and resulted in 10 network clusters. Genes belonging to each of the 10 sub-network are displayed in Table 7.5.

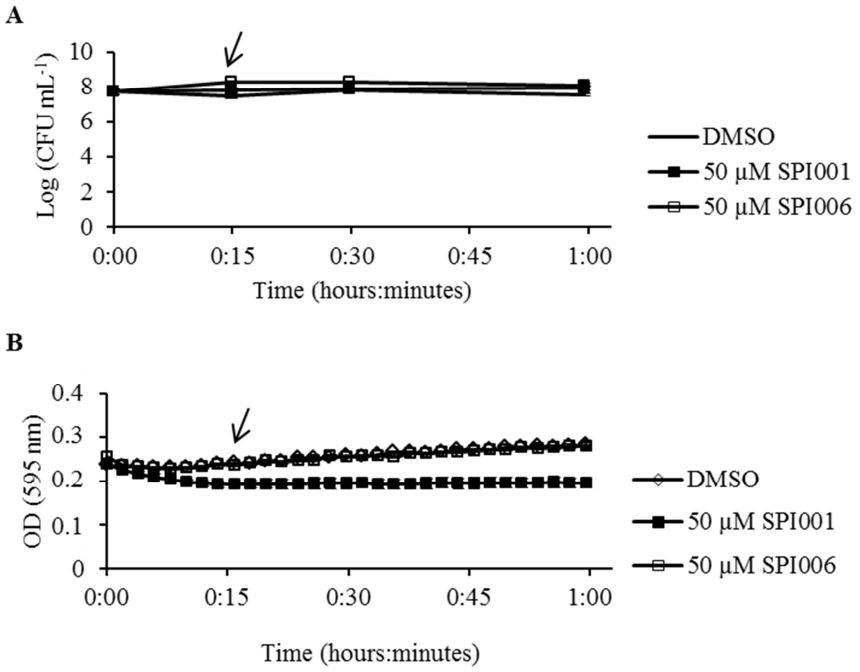


Figure 7.11: Evolution of viability and growth of cells treated with SPI001, SPI006 or DMSO. Stationary phase cells were diluted 100-fold into fresh medium. At OD_{595} = 0.2 (time 0 hours in the figure), cells were treated with 50 µM SPI001, 50 µM SPI006 or 1 % DMSO after which **A.** the number of viable cells and **B.** growth was monitored by counting viable cells and measuring optical density at 595 nm using an automated plate reader respectively. Data points correspond to the mean of three independent repeats. Error bars represent SEM. Samples for RNA isolation were taken 15 minutes after treatment start (indicated by an arrow).

Expression of genes belonging to the first seven sub-networks was upregulated. Sub-networks 1, 2 and 3 contain genes with a function in antibiotic resistance. Genes included in the first two sub-networks encode constituents of two different efflux pumps, supportive of active efflux of SPI001. Sub-network 3 contains genes belonging to the *arnBCADTEF*-PA3559 operon, playing a role in conferring tolerance upon sensing cationic antimicrobial peptides by modifying the lipid A moiety of lipopolysaccharide (LPS). These data suggest a cationic nature of SPI001. In addition to antibiotic resistance mechanisms, genes with a role in oxidative stress protection are upregulated (sub-network 6). Furthermore, increased

expression of genes mainly involved in fatty acid degradation was detected (sub-network 4), suggesting a high amount of available fatty acids upon treatment of cells with SPI001. Also genes classified into the functional group 'Ribosome' are upregulated, which indicates an increased translation rate in SPI001-treated cells. Lastly, expression of a few genes with a role in 'Glycine, serine and threonine metabolism' was increased.

Expression of genes belonging to sub-networks 8, 9 and 10 was downregulated upon treatment with SPI001. These sub-networks contain genes involved in a type VI secretion system, phenazine biosynthesis and protein secretion and motility & attachment respectively, pointing to downregulation of non-essential processes.

Table 7.5: Genes differentially expressed (log2 fold change ≥ 1) upon treatment with SPI001 for each sub-network

Gene number	Gene name	Product name and/or function	Fold change (log2)
		UPREGULATED SUBNETWORKS	
Sub-network 1: Multidrug resistance			
PA14_60820	*oprJ*	outer membrane protein OprJ	2.39
PA14_60850	*mexC*	multidrug efflux RND membrane fusion protein	2.91
PA14_60860	*nfxB*	transcriptional regulator NfxB	2.54
Sub-network 2: Transport of small molecules; Antibiotic resistance and susceptibility			
PA14_23520		MFS transporter	1.04
PA14_23530	*mdr*	secretion protein	1.12
PA14_68110		transcriptional regulator	1.16
PA14_68120	*opmG*	outer membrane protein	1.03
PA14_68130	*emrA*	multidrug resistance protein	1.15
PA14_68140	*emrB*	drug efflux transporter	1.04
Sub-network 3: Amino sugar and nucleotide sugar metabolism			
PA14_18350	*fmt or arnA*	bifunctional UDP-glucuronic acid decarboxylase/UDP-4-amino-4-deoxy-L-arabinose formyltransferase	1.13

Table 7.5 - continued

Gene number	Gene name	Product name and/or function	Fold change (log2)
PA14_18360	*pmrF or arnC*	glycosyl transferase family protein	1.39
PA14_18370	*arnB*	UDP-4-amino-4-deoxy-L-arabinose-oxoglutarate aminotransferase	1.28
PA14_38350	*galU*	UTP-glucose-1-phosphate uridylyltransferase	1.06
PA14_38360	*ugd*	nucleotide sugar dehydrogenase	1.00
Sub-network 4: Valine, leucine and isoleucine degradation; Fatty acid degradation; Butanoate metabolism			
PA14_01620	*aptA*	beta alanine--pyruvate transaminase	2.27
PA14_06600		acyl-CoA dehydrogenase	2.43
PA14_06640		acyl-CoA dehydrogenase	2.70
PA14_13090		acyl-CoA thiolase	1.83
PA14_18140	*mmsB*	3-hydroxyisobutyrate dehydrogenase	-1.02
PA14_25080	*fadB*	multifunctional fatty acid oxidation complex subunit alpha	2.14
PA14_25090	*fadA*	3-ketoacyl-CoA thiolase	2.33
PA14_25840		electron transfer flavoprotein-ubiquinone oxidoreductase	1.80
PA14_25860	*etfB*	electron transfer flavoprotein subunit beta	1.29
PA14_25880	*etfA*	electron transfer flavoprotein subunit alpha	1.11
PA14_41950		enoyl-CoA hydratase	1.37
PA14_42080	*fadB*	3-hydroxyacyl-CoA dehydrogenase	1.02
PA14_42090		acetyl-CoA acetyltransferase	1.09
PA14_66350		acyl-CoA dehydrogenase	1.15
PA14_66840	*phaC2*	poly(3-hydroxyalkanoic acid) synthase 2	-1.07
Sub-network 5: Ribosome			
PA14_08720	*rplK*	50S ribosomal protein L11	1.13
PA14_08730	*rplA*	50S ribosomal protein L1	1.24

Table 7.5 - continued

Gene number	Gene name	Product name and/or function	Fold change (log2)
PA14_08740	*rplJ*	50S ribosomal protein L10	1.35
PA14_08750	*rplL*	50S ribosomal protein L7/L12	1.17
PA14_08860	*rplD*	50S ribosomal protein L4	1.05
PA14_08870	*rplW*	50S ribosomal protein L23	1.12
PA14_08880	*rplB*	50S ribosomal protein L2	1.02
PA14_08890	*rpsS*	30S ribosomal protein S19	1.01
PA14_08900	*rplV*	50S ribosomal protein L22	1.02
PA14_08910	*rpsC*	30S ribosomal protein S3	1.00
PA14_08920	*rplP*	50S ribosomal protein L16	1.07
PA14_08930	*rpmC*	50S ribosomal protein L29	1.07
PA14_08940	*rpsQ*	30S ribosomal protein S17	1.11
PA14_08950	*rplN*	50S ribosomal protein L14	1.14
PA14_08960	*rplX*	50S ribosomal protein L24	1.16
PA14_08970	*rplE*	50S ribosomal protein L5	1.14
PA14_08980	*rpsN*	30S ribosomal protein S14	1.02
PA14_09000	*rplF*	50S ribosomal protein L6	1.00
PA14_09010	*rplR*	50S ribosomal protein L18	1.12
PA14_09020	*rpsE*	30S ribosomal protein S5	1.00
PA14_17060	*rpsB*	30S ribosomal protein S2	1.10
PA14_17070	*tsf*	elongation factor Ts	1.18
PA14_23330	*rpsA*	30S ribosomal protein S1	1.13
PA14_65150	*rplI*	50S ribosomal protein L9	1.12
PA14_67260		histidine/phenylalanine ammonia-lyase	-1.46
PA14_67280	*proW*	ABC transporter permease	-1.02
PA14_67300	*prfoX*	ABC transporter substrate-binding protein	-1.46
PA14_67560	*typA*	GTP-binding protein TypA	1.19
PA14_67600	*glnA*	glutamine synthetase	1.04
PA14_71650	*aspA*	aspartate ammonia-lyase	1.71
Sub-network 6: Adaptation, Protection			
PA14_27220	*ohr*	organic hydroperoxide resistance protein	1.26

Table 7.5 - continued

Gene number	Gene name	Product name and/or function	Fold change (log2)
PA14_27520	*gpo*	glutathione peroxidase	3.38
PA14_27530		MarR family transcriptional regulator	2.66
Sub-network 7: Glycine, serine and threonine metabolism			
PA14_32985	*gcvH2*	glycine cleavage system protein H	2.99
PA14_33000	*gcvP2*	glycine dehydrogenase	2.46
PA14_33010	*glyA2*	serine hydroxymethyltransferase	1.73
DOWNREGULATED SUBNETWORKS			
Sub-network 8: Type VI secretion system			
PA14_33990		ClpA/B-type protease	-1.60
PA14_34010	*hsiG3**	hypothetical protein	-1.67
PA14_34030	*hcp3**	hypothetical protein	-1.60
PA14_34050	*hsiC3**	hypothetical protein	-1.64
PA14_34070	*hsiB3**	hypothetical protein	-1.49
PA14_34080	*lip3**	hypothetical protein	-1.38
PA14_34100	*hsiJ3**	hypothetical protein	-1.35
PA14_34110	*dotU3**	hypothetical protein	-1.29
PA14_34130	*icmF3**	hypothetical protein	-1.44
Sub-network 9: Phenazine biosynthesis			
PA14_09440	*phzE1*	phenazine biosynthesis protein PhzE	-1.44
PA14_39880	*phzG2*	pyridoxamine 5'-phosphate oxidase	-1.85
PA14_39945	*phzC2*	phenazine biosynthesis protein PhzC	-2.00
PA14_39960	*phzB2*	phenazine biosynthesis protein	-1.46
PA14_39970	*phzA2*	phenazine biosynthesis protein	-1.99
Sub-network 10: Protein secretion/export apparatus; Motility & Attachment			
PA14_55780		two-component sensor	-1.21
PA14_55790		hypothetical protein	-1.42
PA14_55850		pilus assembly protein	-1.24

Table 7.5 - continued

Gene number	Gene name	Product name and/or function	Fold change (log2)
PA14_55860		hypothetical protein	-1.48
PA14_55880		hypothetical protein	-1.81
PA14_55890	*cpaF2*	type II secretion system protein	-1.55
PA14_55900		hypothetical protein	-1.22
PA14_55920		type II secretion system protein	-1.93
PA14_55930		pilus assembly protein	-1.85

*As described in Lesic *et al.* (2009)

7.4 Discussion

In this Chapter, we aimed to further characterize the anti-persister compound SPI001 towards its activity on clinical isolates and its combination spectrum with chemical agents. Furthermore, experiments were conducted to unravel the mode of action of SPI001, which will greatly help to gain insight into the persistent state and to develop more targeted therapies in the future.

7.4.1 Biological activity of SPI001

Depending on the site of infection, *P. aeruginosa* encounters different stresses related to the host environment. In addition, for each type of infection, different treatment regimens are recommended[17]. This results in isolates specifically adapted to the conditions experienced during infection and subsequent treatment. Therefore, we tested the activity of SPI001, in combination with conventional antibiotics, on clinical isolates obtained from different environments. The results show that SPI001 is active against all isolates tested, making it a good starting point for the development of an anti-persister therapy.

[17] http://www.uzleuven.be/antibioticagids/

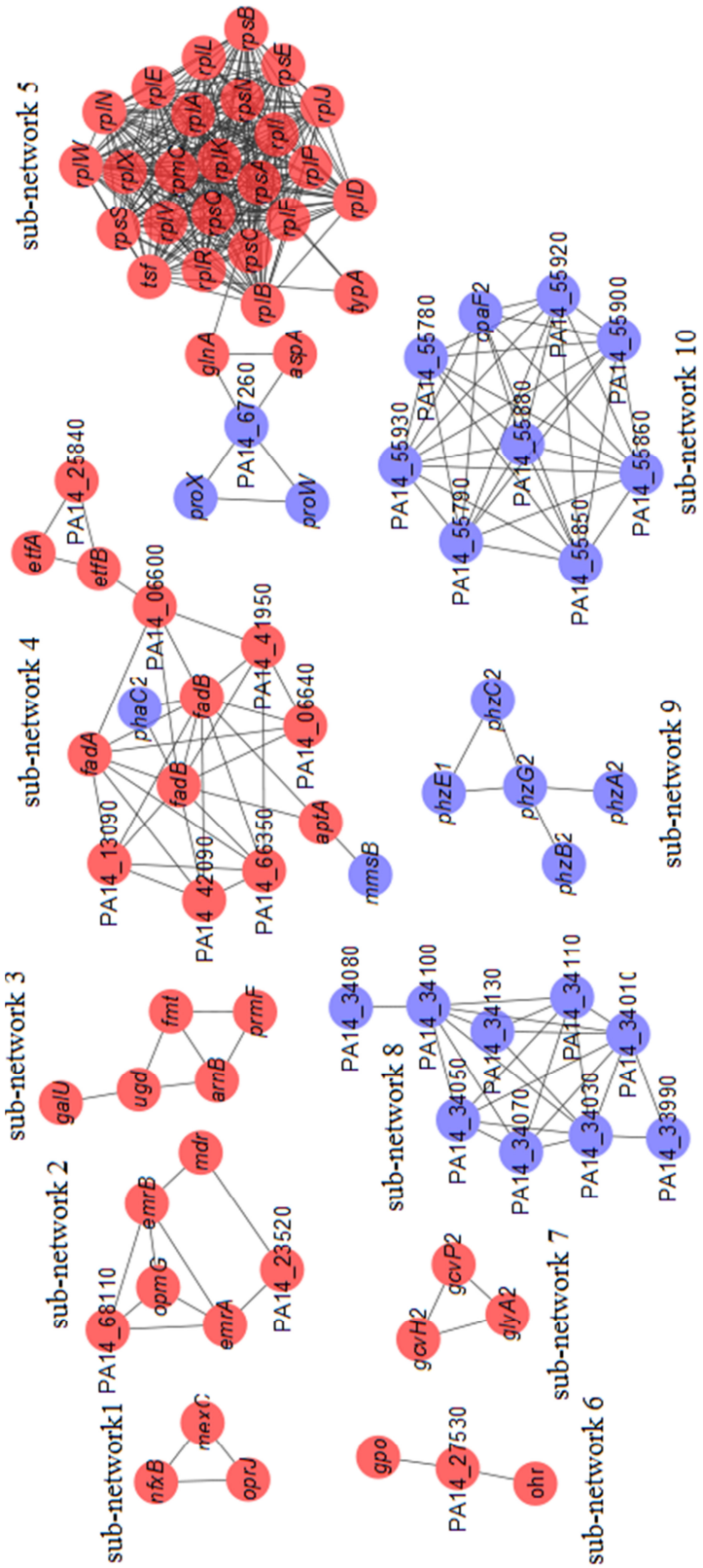


Figure 7.12: Differentially expressed genes mapped on the functional interaction network STRING. Dark blue and red coloured circles represent genes that are either down- or upregulated upon treatment with SPI001 (log2 fold change ≥ 1), respectively. The annotated sub-networks are numbered, gene names or PA14 gene numbers are shown.

To further characterize the combination possibilities of SPI001, we performed synergy testing with 240 different compounds that target specific cellular pathways[18]. Although several β-lactams are present in the Phenotype MicroArrays, none of them was picked up in our experiment. This class of antibiotics is only active against fast-growing cells (Tuomanen *et al.*, 1986), explaining their inactivity on the stationary phase cultures used in our experimental set-up. The fluoroquinolone ofloxacin and several protein synthesis inhibiting antibiotics were picked up (Table 7.3). This confirms our previously obtained results (see Section 6.3.4), namely that combination of SPI001 with ofloxacin and the aminoglycoside amikacin perform better compared to monotreatment with antibiotics. Interestingly, the antibiotic polymyxin B which exerts membrane damage, was also identified during this screening. Polymyxin B belongs to the polymyxins, a class of antibiotics abandoned as systemic therapy in the seventies because of toxicity. However, they are increasingly used nowadays as a last-line antibiotic for the treatment of multi-drug resistant Gram-negative bacteria such as *P. aeruginosa* (Vaara, 2010). Several studies are ongoing to develop less toxic and/or more potent polymyxin derivatives (Vaara, 2013).

In addition, we identified several antiseptics of which the activity improved upon simultaneous administration with SPI001. When confirming these results at the level of a decrease of CFUs using plate counts, only the combination of SPI001 with acriflavine hydrochloride showed improved activity over monotreatment with the antiseptic. Acriflavine is used as a 0.1 % antiseptic solution for wound treatment, although it has been reported to cause skin irritation (Leelavathi *et al.*, 2011). Administration together with SPI001 lowers the active concentration of acriflavine needed, potentially diminishing the side effects associated with the use of this topical antiseptic.

Two other antiseptics, hexachlorophene and ferric chloride, were identified. However, the observed effect evaluated by monitoring regrowth of treated cells was not confirmed when assessing the effect with viable cell counting (Figure 7.7). The reason for this discrepancy is at present unclear.

[18] www.biolog.com/products-static/phenotype_microbial_cells_use.php

7.4.2 Mode of action of SPI001

SPI001 is able to exert membrane damage at relatively low concentrations. At a SPI001/lipid molar ratio of 1, which corresponds to a SPI001 concentration of 25 µM (= 1/8 X MIC value), 50 % leakage is achieved in SUVs. For comparison, 50 % leakage by polymyxin B is induced at 1 µg mL^{-1} (1.6 X MIC). Disruption of the lipid bilayer by SPI001 was detected in both the bacterial and eukaryotic model. SPI005, a chemical analogue that, just like SPI001, displays an anti-persistence effect but no bactericidal effect on stationary phase cells, caused less leakage in the eukaryotic system as compared to the bacterial model. Bacteria contain anionic phospholipids in their membranes while the eukaryotic ones are composed of neutrally charged components (Epand & Epand, 2009) as reflected in our phospholipid models. Possibly, SPI005 requires the negative charge present in the bacterial model to exert its membrane disrupting capacity. The ability of SPI001 to disrupt lipid bilayers provides a plausible explanation for the cidal effect on persister cells. The antibiotic tolerance of persister cells is typically attributed to their metabolic quiescent, dormant state in which the antibiotic targets are less active (Wood *et al.*, 2013). However, the lipid bilayer remains vulnerable to membrane-disrupting agents.

To analyze transcriptional changes induced upon exposure of growing cells to SPI001, an RNA-Seq experiment was performed. Network analysis showed that several genes involved in antibiotic resistance were upregulated. First of all, the expression of genes encoding two different efflux pumps was increased, indicating that SPI001 is actively extruded. Genes encoding the efflux pump MexCD-OprJ are upregulated (sub-network 1). This efflux pump is inactive under standard laboratory conditions but it was shown that expression is induced by membrane-acting agents (Fraud *et al.*, 2008). Genes encoding a second multidrug efflux pump, EmrAB-OpmG, were also upregulated (sub-network 2). This efflux pump is not yet characterized in *P. aeruginosa* but the genes show homology to the ones of the *E. coli* EmrAB-TolC efflux pump (Tanabe *et al.*, 2009). This efflux pump was shown to confer resistance to hydrophobic toxins in *E. coli* (Lomovskaya & Lewis, 1992). Secondly, *ugd* and *arnA-C*, genes involved in a tolerance conferring pathway, were upregulated (sub-network 3). They are involved in synthesis of undecaprenol-phosphate-linked 4-amino-4-deoxy-L-arabinose (L-Ara4N). This molecule serves as a carrier of L-Ara4N which is added to the LPS component lipid A as a response to cationic peptide stress. In this way, the negative charge of

LPS is reduced, conferring increased tolerance against cationic peptides such as for instance polymyxin B (Moskowitz *et al.*, 2004). Other genes upregulated in response to SPI001 exposure include those involved in oxidative degradation of fatty acids (sub-network 4). Fatty acid degradation in *P. aeruginosa* occurs by repetition of a sequence of four reactions which shortens the fatty acid by two carbon atoms in each round. The successive steps are carried out by an acyl CoA dehydrogenase, an enoyl CoA hydratase, a 3-hydroxyacyl COA dehydrogenase and a thiolase respectively (Yuan *et al.*, 2012). Upregulation of fatty acid degradation may point to the high abundance of free fatty acids, possibly resulting from membrane damage. The latter may lead to oxidative stress (Dominguez-Cuevas *et al.*, 2006), explaining the upregulation of genes involved in oxidative stress tolerance (sub-network 6). The largest functional group of upregulated genes, sub-network 5, contains members encoding 50S and 30S ribosomal proteins. In general, upregulation of ribosomal proteins mostly results from treatment with a protein synthesis inhibitor (Davies *et al.*, 2006). However, if SPI001 acts on protein synthesis, additional changes are expected such as changes in genes involved in amino acid biosynthesis or heat shock genes (Davies *et al.*, 2006; Morita *et al.*, 2014). Therefore, we hypothesize that upregulation of these ribosomal proteins is necessary to support production of the gene products encoded by the upregulated genes such as those involved in efflux pumps and fatty acid metabolism.

A number of genes, belonging to non-essential processes, are downregulated. Likely, shutting down these non-essential processes saves energy which can be used to counteract the stress experienced during treatment with SPI001. Sub-network 8 contains genes encoding proteins of the third type VI secretion system of *P. aeruginosa*, also named HSI-III. So far, only the function of the first type VI secretion system is understood, and is involved in bacterial competition and pathogenesis (Filloux *et al.*, 2008). Also expression of genes involved in motility and biosynthesis of phenazines are downregulated. These results are also indicative of downregulation of virulence, another advantage of treatment with SPI001 (see Section 1.4.3.1).

Based on our results, it is likely that SPI001 acts by disrupting the membrane. One of the mechanisms *P. aeruginosa* uses to gain tolerance against membrane-acting agents is by active extrusion of the compound (Ramos *et al.*, 2002). This may explain the absence of SRM formed after exposure of the efflux mutant to SPI001. The *P. aeruginosa* mutant lacks 4

major efflux pumps (Morita *et al.*, 2001), including MexCD-OprJ which was implicated in tolerance against membrane-acting agents (Fraud *et al.*, 2008). However, when treating *E. coli* with SPI001, no SRM were formed either. Possibly, several mutations need to accumulate to decrease susceptibility against SPI001. Therefore, a more gradual evolution experiment may be more successful to obtain SRM against SPI001.

7.5 Concluding remarks

In this Chapter, we showed that SPI001 is active against different clinical isolates and acts in combination with a variety of antibiotics and antiseptics, hereby further increasing its potential as anti-persister molecule. Furthermore, it was shown that SPI001 likely acts by disrupting membranes, which provides a plausible explanation for its cidal action on persister cells. Experiments to confirm the proposed mode of action are needed. In addition, assessment of the combination therapy under more *in vivo* like conditions will shed more light on the potential of SPI001 as a supplement to conventional antibiotic therapy.

Chapter 8

General conclusions and perspectives

Pseudomonas aeruginosa is an opportunistic pathogen, causing severe infections in (partially) immunocompromised persons. On top of its considerable intrinsic resistance, this bacterium is able to acquire additional resistance mechanisms which can lead to development of strains resistant to nearly all available antibiotics (Breidenstein *et al.*, 2011). However, even in the absence of resistance, infections are often difficult to eradicate. One of the major factors held responsible for the refractory nature of such infections is the presence of persister cells (Mulcahy *et al.*, 2010; Fauvart *et al.*, 2011). These antibiotic tolerant cells comprise a small fraction of a genetically heterogeneous population which survives the cidal effect of antibacterial agents. Once the antibiotic pressure drops, persisters are able to start growing again and form a susceptible population, hereby causing relapse of infection. In this way, the presence of the infecting strain in the human body is maintained, increasing the probability of developing resistance against the used antibiotic treatment (Cohen *et al.*, 2013). Targeting persisters provides a good strategy to prevent relapse of infection, avoiding development of chronic infections which negatively affect the outcome of patients.

Design of a rational treatment strategy is difficult since the mechanisms underlying persistence in *P. aeruginosa* are not yet understood. Hence, a screen for mutants displaying an altered persister fraction was carried out in our research group previously (De Groote *et al.*, 2009). In this thesis, we followed up on this research by studying the newly identified gene *dnpA*, which represents a possible target for the rational design of an anti-persister strategy. Since several mechanisms underlie the formation and/or maintenance of persister cells, development of a rational anti-persister therapy is challenging. In a second part we used a top-down approach to identify anti-persister molecules which can be used in combination with conventional antibiotics, providing a potential starting point for development of an anti-persister strategy.

8.1 The newly identified persistence gene *dnpA*

In a first part of this thesis, the previously uncharacterized persistence gene, which we named *dnpA* (de-*N*-acetylase involved in persistence; gene locus PA14_66140/PA5002), was studied. Our results show that *dnpA* is a *bona fide* persistence gene since fluoroquinolone tolerance of a *dnpA* mutant is strongly reduced both in planktonic culture and in a biofilm model whereas overexpression of *dnpA* in the wild-type strain increases the persister fraction. *dnpA* is part of the conserved lipopolysaccharide (LPS) core oligosaccharide biosynthesis gene cluster, however, no obvious role in LPS biosynthesis could be demonstrated. Possibly, DnpA is responsible for minor LPS modifications which are not detected by the LPS profiling methodology used here, such as for example changes in the acetylation state of LPS (Slauch *et al.*, 1995). Alternatively, DnpA could be involved in monitoring the bacterium's environment. It was reported that the gene upstream of *dnpA*, PA14_66150, is involved in sensing cationic antimicrobial peptides which leads to the induction of the LPS modification operon *arnBCADTEF*-PA3559 (Jochumsen *et al.*, 2011). The latter is responsible for adding L-Ara4N to lipid A, hereby decreasing the susceptibility to cationic peptides (Moskowitz *et al.*, 2004). Since PA66150-*dnpA* is conserved as one unit in all of the genomes analyzed (Figure 4.4), it is possible that *dnpA*, together with PA14_66150, has a role in monitoring LPS biosynthesis/assembly or outer membrane physiology (Jochumsen *et al.*, 2011). This is supported by our transcriptome analysis, which indicated that DnpA affects the expression of genes involved in cell surface-associated processes. In support of these results we showed that DnpA is anchored within the inner membrane with C-terminal part, which includes the catalytic domain, located at the cytoplasmic side of the membrane. As such, DnpA is perfectly located to fulfill a role in membrane-associated processes. The *dnpA* mutant did not show an altered susceptibility towards polymyxin B, indicating that *dnpA* may be involved in other surface-related processes. No difference was seen between LPS profiles or outer membrane fractions of wild-type and *dnpA* mutant cells. This does not exclude minor modifications of membrane proteins or changes in membrane composition itself. For instance, as a follow-up study, the structure of LPS extracted from both wild type and *dnpA* mutant can be evaluated (Choudhury *et al.*, 2005) as well as fatty acid and polar lipid composition.

How exactly persistence can be regulated by sensing the extracellular environment remains unknown. One possibility is provided by the phenomenon known as contact dependent growth inhibition (CDI), in which bacteria regulate their growth rate by cell contact (as

reviewed by Ruhe *et al.* (2013)). In *Escherichia coli*, this mechanism is regulated by a two-partner secretion system in which a ß-barrel protein exports a toxin across the outer membrane. Upon contact with the target cell via a receptor, the toxin is cleaved and translocated into the target cells where it decreases the proton motive force leading to cessation of growth. Also in stationary phase cultures, a form of CDI was observed (Lemonnier *et al.*, 2008). CDI expression is activated at high cell densities when cell-cell contact is more common (Lemonnier *et al.*, 2008; Ruhe *et al.*, 2013), showing similarities with the kinetics of persister formation in which the persister level starts increasing in mid-to late-exponential phase (Keren *et al.*, 2004a).

Based on sequence analysis, we predicted that DnpA is a de-*N*-acetylase, acting on a yet unidentified substrate. Site-directed mutagenesis suggests that this enzymatic activity is essential for DnpA-mediated persistence. In the future, more information about the putative substrate specificity of DnpA could be obtained using a glycan array (Blixt *et al.*, 2004). Based on the obtained results, biochemical *in vitro* activity can be tested in more detail as described in Deli *et al*. (2010). Here, deacetylation is quantified using the molecule fluorescamine, which becomes fluorescent upon binding the free amino groups resulting from enzymatic de-*N*-actylase activity. In addition, it has been shown that activity of other members of the PIG-L family can be inhibited by small molecules, without affecting human PIG-L members (de Macedo *et al.*, 2003; Metaferia *et al.*, 2007; Gammon *et al.*, 2010). The inhibitory effect of existing inhibitory molecules can be tested *in vitro*, followed by evaluation of its effect on whole cells. Lastly, since multiple mechanisms seem to be involved in the formation of persister cells, it should be tested whether absence of *dnpA* (activity) is sufficient to obtain more effective eradication of the infecting population *in vivo*.

8.2 The anti-persister molecule SPI001

In a second part of this thesis we identified and characterized the promising anti-persister molecule SPI001 in detail. This small molecule is able to strongly reduce the persister fraction of *P. aeruginosa* lab strains and several different clinical isolates in combination with mechanistically different classes of antibiotics. Moreover, this compound was also able to reduce the persister fraction of other clinically relevant bacteria such as *E. coli*, *S.* Typhimurium and *Staphylococcus aureus*. Taken together, SPI001 has the potential to be

developed into an anti-persister therapy. An important factor for further development is its activity in an *in vivo* infection model. Currently, a first experiment (carried out by Valerie Defraine, in collaboration with Prof. Françoise van Bambeke, UCL) in which activity of SPI001 in combination with ofloxacin is tested on *P. aeruginosa* infected human THP-1 cells, showed promising results. In contrast to monotreatment with ofloxacin, the combination of ofloxacin with SPI001 is able to completely eradicate the infecting *P. aeruginosa* cells without significantly affecting the viability of the eukaryotic cells (unpublished results). A next step would be to test this combination therapy in a more complex *in vivo* infection model. Since persisters play an important role in chronic infections, it is important to assess the anti-persister therapy in a chronic infection model, of which only a few are available. A first model is represented by the chronic *P. aeruginosa* mouse airway infection model. Here, the mouse lung pathology resembles that of chronically infected CF patients (Facchini *et al.*, 2014). A second model is the *S. aureus* mediated deep-seated thigh infection model in neutropenic mice which resembles a chronic infection in immunocompromised patients (Conlon *et al.*, 2013). A third possibility is provided by the *S.* Typhimurium intragastric mouse infection model, in which the presence of antibiotic-tolerant populations was shown upon treatment with ciprofloxacin, causing relapse of infection after discontinuation of antibiotic therapy (Kaiser *et al.*, 2014).

Currently, a relatively high concentration of SPI001 is needed to reduce the persister fraction *in vitro*. It is unlikely that such high concentrations can be reached within the infected tissues upon systemic application. Future steps may involve optimization of the SPI001 structure to enhance activity. A recent research paper points to the possibility to optimize each separate part of a molecule to obtain the most potent variant, as performed in the case of the anti-mycobacterial 4-aminoquinolone piperidine amides (Naik *et al.*, 2014). *P. aeruginosa* is also known to infect surgical sites and burn wounds (Altoparlak *et al.*, 2004; ECDC, 2013). This makes SPI001 a potential candidate for use as topical agent, allowing much higher compound concentrations at the site of infection (Kaye, 2000; Spann *et al.*, 2003). *P. aeruginosa* is also able to form biofilms on indwelling devices such as catheters and endotracheal tubes giving rises to severe infections (Bouza *et al.*, 2004; Gil-Perotin *et al.*, 2012; Cole *et al.*, 2014). For intravascular catheter-related infections, SPI001 can be used in the so called 'antibiotic lock therapy' (ALT) in which very high concentrations of antibiotic are left within the catheter (Mermel *et al.*, 2009). As SPI001 likely acts on the bacterial membrane, it would also be an excellent candidate for coating the surface of indwelling devices (Zelichenko *et al.*, 2013). As

is the case with ALT, high concentrations of the coated agent can be reached, circumventing possible concentration-related problems of SPI001.

In this thesis, we showed that SPI001 likely acts by inducing membrane damage by disrupting the lipid bilayer, causing leakage of carboxyfluorescein encapsulated in SUV. However, some membrane-acting agents can exert their action without disrupting the lipid bilayer. Examples include peptides or molecules that incorporate into the membrane and cause depolarization (Eun *et al.*, 2012) or delocalize peripheral membrane proteins (Wenzel *et al.*, 2014). To further confirm our hypothesis, the membrane damaging effect should be tested on whole cells, for example by a potassium leakage assay (Wenzel *et al.*, 2014). In addition, it has been shown that membrane disrupting properties rely on the composition of the lipid bilayer, which is different among Gram-positive and Gram-negative bacteria (Randall *et al.*, 2013). Therefore, the membrane-disrupting hypothesis should also be tested on Gram-positive bacteria. In addition, it would also be interesting to test the activity on *Mycobacterium*. It was shown that the inner membrane is unusually rich in diacyl phosphatidylinositol dimannosides, creating a bilayer environment with very low fluidity, which possibly has an impact on the activity of SPI001 (Bansal-Mutalik & Nikaido, 2014).

Overall, our screening identified a very promising compound which in combination with conventional antibiotics leads to more potent treatment of bacterial populations. Therefore, it might be useful to screen other libraries for similar compounds. However, some limitations and drawbacks are associated with the used screening method. First of all, quantification of the effect on persistence is influenced by effects on the regrowth of the treated cultures. Viable cell counting experiments remain the most accurate way to assess the number of persisters although they are time consuming. Secondly, only 200 compounds could be screened in 24 hours, making compound discovery a slow process. To increase throughput, the use of a metabolic dye seems a good alternative. This strategy was reported in literature in the search of potentiators of antifungals (Lafleur *et al.*, 2013). Compounds that lower the metabolic activity are also identified while they do not necessarily affect the number of surviving persister cells. Therefore, plate counting experiments, in first instance by spot plating, remain necessary to confirm the observed effect.

8.3 Concluding remarks

This work has contributed to the characterization of *dnpA*, a newly identified persistence gene in *P. aeruginosa* which affects persister levels both in free-living and biofilm state. Although the mechanism is not yet fully understood, it seems that *dnpA* affects surface-associated processes, which represents a novel mechanism underlying antibiotic tolerance. Future experiments will reveal if targeting this specific gene significantly reduces persistence in *vivo*. In addition, we identified a very promising anti-persister compound acting in combination with different classes of antibacterial agents and, importantly, showing activity against both Gram-negative and Gram-positive pathogens. The work presented in this thesis shows that alternative treatment strategies may improve the outcome of current treatments. General awareness of the pressing need for new antibiotics has increased over the last years and action is being undertaken both at the governmental level and in industry. For instance, the European government initiated several initiatives in the framework of Horizon 2020 to support identification and further development of new antibacterial agents[19]. Also, important pharmaceutical companies like Roche are re-investing into antibacterial research[20]. Overall, there is hope to find new antibacterials which can be used in improved treatment strategies to keep ahead of the threat posed by infectious diseases.

[19] http://ec.europa.eu/programmes/horizon2020/

[20] http://www.nature.com/nrd/journal/v13/n3/pdf/nrd4273.pdf

Addendum I

Identification and characterization of an anti-pseudomonal dichlorocarbazol derivative displaying anti-biofilm activity[21]

I.1 Contribution of authors to the experimental work

Screening of the compound library was carried out by V. Liebens, W. J. Knapen and T. Swings. Evaluation of growth inhibition on bacterial free-living and biofilm cultures was conducted by H. P. Steenackers and S. Robijns (*Escherichia coli* and *Porphyromonas gingivalis*), A. Lippell and A. J. O'Neill (*Staphylococcus aureus* and *Staphylococcus epidermidis*), E. Gerits and S. Beullens (*Pseudomonas aeruginosa*). Microscopic experiments described in this Chapter were performed by A. Krona and M. Lövenklev, cell viability assays were carried out by M. Veber and M. Fröhlich.

I.2 Introduction

P. aeruginosa is an opportunistic pathogen that primarily infects cystic fibrosis (CF) patients and immunocompromised individuals such as HIV and burn wound patients but also frequently causes nosocomial infections (Kerr & Snelling, 2009). Up to 80 % of CF patients get infected by this pathogen, making it one of the dominant bacteria in the CF lung. Mostly,

[21] This Chapter was published as: Liebens, V., Gerits**,** E., Knapen, W. J., Swings, T., Beullens, S., Steenackers, H. P., Robijns, S., Lippell, A., O'Neill, A. J., Veber, M., Fröhlich, M., Krona, A., Lövenklev, M., Corbau, R., Marchand, A., Chaltin, P, De Brucker, K., Thevissen, K., Cammue, B., Fauvart M., Verstraeten, N., Michiels J. (2014). Identification and characterization of an anti-pseudomonal dichlorocarbazol derivative displaying anti-biofilm activity. *Bioorganic & Medicinal Chemistry Letters*, **24**, 5404-5408.

P. aeruginosa establishes a chronic infection in the CF airway that significantly contributes to the mortality of these patients (Folkesson *et al.*, 2012). In addition, according to a recent report from the European Centre for Disease Prevention and Control, *P. aeruginosa* is responsible for 8.9 % of all nosocomial infections in Europe, making this pathogen one of the leading causes of healthcare-associated infections (ECDC, 2013). *P. aeruginosa* infections are extremely difficult to treat with currently available antibacterials. This is primarily due to significant intrinsic resistance, caused by low permeability of the outer cell membrane (Strateva & Yordanov, 2009). Additionally, *P. aeruginosa* has a remarkable capacity to acquire additional resistance mechanisms. This has led to development of resistance towards all available classes of antibiotics (Breidenstein *et al.*, 2011) resulting in rapidly rising rates of (multi)drug resistance (Obritsch *et al.*, 2004). The number of new antibiotics in the pharmaceutical pipeline active against *P. aeruginosa* is very limited (Bassetti *et al.*, 2011; Pendleton *et al.*, 2013) underlining the pressing need for the development of new antipseudomonal agents. In this Chapter, the identification and characterization of a promising new anti-pseudomonal small molecule is described

I.3 Experimental procedures

Bacterial and human cell culture conditions. *Pseudomonas* strains, *E. coli* TG1 and *Staphylococcus* species were grown in 1:20 Trypticase Soy Broth (TSB) at 37 °C, shaking at 200 rpm. *P. gingivalis* was cultured statically in an anaerobic jar ($H_2/CO_2/N_2$; 5:5:90 %, Anoxomat AN2OP, Mart® Microbiology BV, Drachten, The Netherlands) at 37 °C. For solidified medium, 1.5 % agar was added. Strains used in this Chapter are listed in Table I.1. Osteoblasts and mesenchymal stromal cells were cultured in Advanced DMEM, 10 % FBS, 1x GlutaMAX, 0.05 mg mL^{-1} gentamicin. Microvascular endothelial cells were grown in Medium 131, Microvascular Growth Supplement, 0.05 mg mL^{-1} gentamicin (all from Gibco, Carlsbad, CA, USA). Human aortic endothelial cells were cultured in M200 medium with Low serum growth supplement (Gibco, Carlsbad, CA).

Small-molecule library and compound storage. See Chapter 6.

Minimal inhibitory concentration (MIC) determination. MIC values were determined as described before (Chapter 4). The lowest antibiotic concentration resulting in the absence of bacterial growth was considered as the MIC.

Table I.1: Bacterial strains used in this Chapter

Strain	Description[a]	Source or reference
P. aeruginosa PA14	Wild type, Km^R	Lee *et al.* (2006)
P. aeruginosa YM	Wild type	Morita *et al.* (2001)
P. aeruginosa YM64	*mexAB-oprM::FRT*, *mexXY::FRT*, *mexCD-oprJ::FRT*, *mexEF-oprN::FRT*	Morita *et al.* (2001)
E. coli TG1	Wild type, [F' *traD36 proAB lacZ ΔM15] supE thi-1 Δ(lac-proAB) Δ(mcrB-hsdSM)5(rK - mK -*	Carter *et al.* (1985)
S. aureus SH1000	Functional *rsbU* derivative of 8325-4 *rsbU*$^+$ (wild type strain cured of prophages)	O'Neill *et al.(2010)*; Horsburgh *et al.*(2002)
S. epidermidis RP62A	Wild type, ATCC 35984	ATCC
P. gingivalis	Wild type, ATCC 33277	ATCC

[a]Km^R, kanamycin resistant

Biofilm inhibition assay. To assess inhibition of biofilm formation, the Calgary biofilm device (Nunc-Immuno TSP, VWR International) was used. Bacterial cells from an overnight culture were diluted 1:200 (*P. aeruginosa*), 1:100 (*E. coli* and *Staphylococcus* species) or 1:10 (*P. gingivalis*) into fresh growth medium. Biofilms were allowed to form for 24 h (*P. aeruginosa*, *E. coli* and *Staphylococcus* species) or 72 h (*P. gingivalis*) on the polystyrene pegs and were quantified by crystal violet staining. The lowest concentration that completely prevented biofilm formation was considered the minimal biofilm inhibitory concentration (MBIC). For *E. coli* and *P. gingivalis*, $MBIC_{90}$ (concentration with 90 % biofilm inhibition) values were calculated. Prevention of *Staphylococcus* biofilm growth was quantified by determining the lowest concentration of compound that completely inhibits biofilms from seeding fresh growth medium as described previously (Miller *et al.*, 2005; Ooi *et al.*, 2010).

Biofilm eradication assay. *P. aeruginosa* stationary phase cells were 200-fold diluted and biofilms were allowed to form on polystyrene pegs as described above. 24 h old biofilms were subjected to treatment with different concentrations of compound during 24 h at 37 °C. Subsequently, biofilm cells were recovered by means of sonication after which the number of surviving cells was determined by plate counting (Harrison *et al.*, 2010).

Microscopic evaluation of biofilms. Overnight *P. aeruginosa* cultures were diluted into fresh TSB to 1 x 10^5 cells mL^{-1} and transferred to a 6-well plate containing a titanium disc and 0, 10 and 32.5 µM of compound, respectively. Subsequently, biofilms were allowed to form for 19 h at 37 °C with agitation. Biofilm formation was analyzed using confocal laser scanning microscopy (CLSM, Leica TCS SP5). In addition, viability of the cells was examined by the LIVE/DEAD®BacLight™ viability kit (Molecular Probes). Each sample was analyzed in triplicate. During CLSM evaluation, 25 digital images were taken with X-Y scan a few µm above the surface plane with an HCX PL APO CS objective with 63 times magnification and numerical aperture 1.20. The thickness of the optic sections was 577 nm at full width half maximum and image size of the micrographs was 2048 x 2048. Image analysis was performed in Mathlab to calculate the fraction of live and dead bacteria.

Cell viability assay. Cell viability of human osteoblasts, bone marrow derived mesenchymal stem cells and microvascular endothelial cells was tested with trypan blue staining according to the ISO 10993-5 standard. Briefly, cells were seeded at 2×10^4 cells cm^{-2} to 96-well plates, and were exposed in four replicates to different conditions. Afterwards, the cell culture medium was removed and 1:3 of trypan blue in DMEM medium was added to the culture for three minutes. Dead (blue) and live (transparent) cells in two visual fields were counted in each of four wells.

Tube formation assay. The surface of 24-well plates was coated with 250 µL of phenol-red free Matrigel (Becton Dickinson, Bedford, MA) per well and human aortic endothelial cells were seeded at 2×10^4 cells cm^{-2}. Cells were grown in different conditions as indicated in the text. Four hours after seeding of the cells, 9 random phase-contrast digital images per treatment were taken with a digital camera (DS-Fi1, Nikon, Tokyo, Japan) using a 10 x objective lens. Tube-like structures were analyzed with ImageJ software (http://rsbweb.nih.gov/ij/) and the average tube length per field was calculated.

I.4 Results and Discussion

I.4.1 Screening for anti-pseudomonal compounds

To identify novel anti-pseudomonal compounds, we conducted a medium-throughput screening of a small compound library comprising 23909 commercially available diverse

compounds. Overnight *P. aeruginosa* PA14 cultures (Table I.1) were diluted into fresh 1:20 TSB and transferred to a 96-well plate containing a single compound per well at a final concentration of 20-50 µM. After 24 h of incubation at 37 °C with agitation, growth inhibition was evaluated by measuring culture turbidity. Compounds that caused > 90 % growth inhibition were selected and retested (fresh sample), after which the most promising compound, 1-(sec-butylamino)-3-(3, 6-dichloro-9H-carbazol-9-yl) propan-2-ol, designated as compound **1** (Figure I.1), was selected for further characterization.

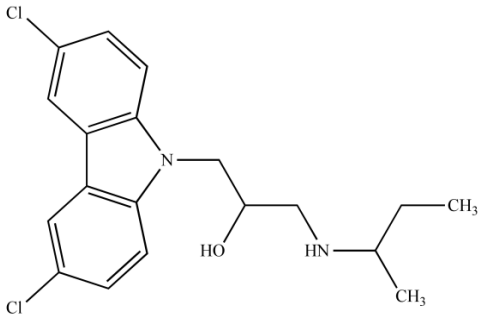


Figure I.1: Schematic structure of compound 1**.**

I.4.2 Structure-activity relationship analysis

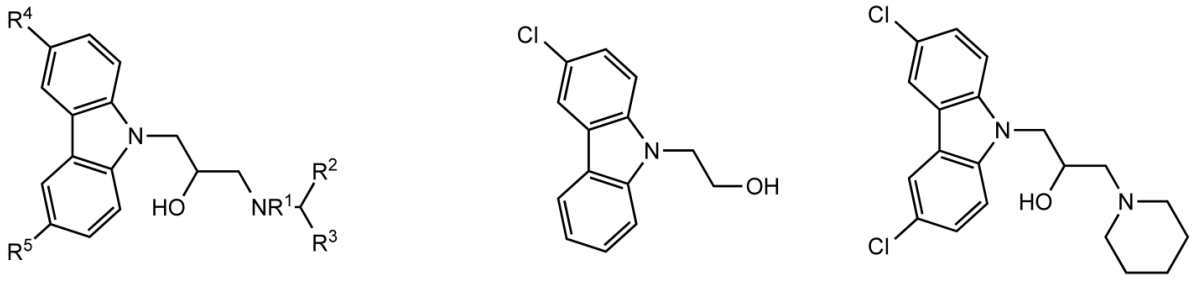
In order to analyze the structural determinants important for pseudomonal growth inhibitory activity of **1,** commercially available chemical analogues of **1** were obtained (compounds **2-8**, Figure I.2). The MIC of these compounds was determined against *P. aeruginosa* grown in 1:20 TSB (Table I.2). The lowest compound concentration resulting in the absence of bacterial growth was considered as the MIC. None of the tested analogs showed better antibacterial activity against *P. aeruginosa* compared to the original hit compound **1** (MIC of 50 µM). The minimal bactericidal concentration (concentration resulting in absence of bacterial regrowth after dilution in Mueller-Hinton Broth (MHB)) of **1** against *P. aeruginosa* is 50 µM, indicating that this compound acts bactericidal.

The MIC of **1** against a *P. aeruginosa* efflux mutant YM64 (Table I.1) lacking all four major *mex* operons for multidrug efflux pumps (Morita *et al.*, 2001) is 12.5 µM, which is significantly lower as compared to its corresponding wild-type strain YM (MIC of 50 µM).

This result indicates that **1** probably has to cross the *P. aeruginosa* outer membrane to exert its action.

Table I.2: Minimal inhibitory concentration (MIC) for N-alkylated 3, 6- dihalogeno-carbazoles against *P. aeruginosa* PA14

Compound	MIC (µM)
1	50
2	50-100
3	>200
4	50-100
5	100-400
6	50-200
7	200-400
8	50-100



(2) R^1: CH_3; R^2: H; R^3: CH_2OH; R^4: Cl; R^5: Cl
(3) R^1: CH_2CH_2OH; R^2: H; R^3: CH_2OH; R^4: Cl; R^5: Cl
(4) R^1: H; R^2: H; R^3: tetrahydrofuran; R^4: Cl; R^5: Cl
(5) R^1: CH_2CH_3; R^2: H; R^3: CH_3; R^4: Cl; R^5: Cl
(8) R^1: CH_3; R^2: H; R^3: H; R^4: Br; R^5: Br

(6)

(7)

Figure I.2: Schematic structure of N-alkylated 3, 6-dihalogenocarbazoles represented by numbers 2-8.

I.4.3 Further characterization of 1

I.4.3.1 Effect on P. aeruginosa clinical isolates

The susceptibility of several clinical isolates obtained from CF and non-CF patients towards **1** was tested. These strains were as susceptible towards **1** as the wild-type strain used in our

study (Table I.3). In addition, isolates resistant towards specific clinically used antibiotics did not show cross-resistance towards **1**. These results indicate that **1** is active against strains adapted to *in vivo* conditions, including antibiotic treatment regimes.

Table I.3: Minimal inhibitory concentration (MIC) of 1 **on *P. aeruginosa* clinical isolates obtained from cystic fibrosis (CF) and non-CF patients.** These strains were kindly provided by Prof. Françoise van Bambeke (Université Catholique de Louvain).

Clinical isolate	Resistance profile*	MIC 1 (µM)
Non-CF isolates		
PA08	ticarcillin	50-100
PA53	ticarcillin	50
PA129	piperacillin	50-100
PA346	ticarcillin, piperacillin	50-100
PA424	ticarcillin	50-100
CF isolates		
PA1250	ticarcillin, cefepime	50
PA1255	ticarcillin, piperacillin, ceftazidime, cefepime	50-100
PA1256	/	25-50
PA1270	/	50
PA1271	ticarcillin	50-100

*Based on EUCAST values.

I.4.3.2 Effect on P. aeruginosa biofilms

In vivo, *P. aeruginosa* is well known to form biofilms, multicellular structures of cells embedded in a self-produced extracellular matrix (Honda, 2011; Romling & Balsalobre, 2012). The bacterial cells residing in these biofilms are significantly less susceptible to antibiotics and host defenses. This complicates treatment and often leads to chronic and recurrent infections (Costerton *et al.*, 1999; Romling & Balsalobre, 2012). We assessed whether **1** is capable of preventing biofilm formation *in vitro* as described in Experimental procedures. The result shows that **1** completely prevents biofilm formation of *P. aeruginosa* at

50 µM (Table I.4). To further evaluate the effect of **1** on biofilm formation on clinically relevant implant material, the formation of *P. aeruginosa* biofilms on smooth titanium discs (10 mm diameter, 2 mm thick) in the presence of **1** was investigated. The results show that **1** is capable of preventing biofilm formation of *P. aeruginosa*, already at a concentration of 10 µM. The total area fraction, defined as the percentage of surface covered by bacteria, was reduced by approximately 50 % upon addition of **1**. Biofilms formed on the titanium discs in the presence of **1** comprised a greater proportion of dead cells within the biofilm compared to the control experiment. This was most evident at the highest concentration of **1** (32.5 µM)(Figure I.3). Taken together, these microscopy results clearly show that **1** prevents biofilm formation of *P. aeruginosa* on titanium discs.

Next, we tested the capability of **1** to eradicate mature biofilms of *P. aeruginosa* grown on polystyrene pegs using the Calgary biofilm device. Treatment of biofilms with 12.5 µM or higher concentrations of **1** resulted in a drastic decrease of viable cells within the biofilm, which demonstrates the capability of **1** to eradicate biofilms (Figure I.4). These data suggest a biofilm-specific effect of **1** in *P. aeruginosa*.

I.4.3.3 Effect on other pathogens

The effect of **1** was subsequently evaluated on the inhibition of growth and biofilm formation of other clinically relevant bacteria listed in Table I.1. The MIC and MBIC values were determined as described above. *E. coli*, *S. aureus* and *S. epidermidis* were selected as they are frequently involved in biofilm-associated infections (Romling & Balsalobre, 2012). Additionally, the efficacy of **1** was evaluated on the obligate anaerobic pathogen *P. gingivalis*, which plays an important role in the initiation of periodontitis, a biofilm-associated infection of the gum eventually leading to tooth loss (Darveau *et al.*, 2012). Results shown in Table I.4 indicate that **1** is capable of inhibiting both planktonic and biofilm growth of other clinically important Gram-negative bacteria such as *E. coli* and *P. gingivalis* at concentrations well below those active against *P. aeruginosa*. Furthermore, **1** has a bacteriostatic and biofilm inhibitory effect on both Gram-positive *S. aureus* and *S. epidermidis* strains. The capability of **1** to target a wide range of bacterial species makes it a potential starting point for the development of a novel broad-spectrum antibiotic. A previous, unrelated screening campaign identified **1** as a candidate antifungal (Thevissen *et al.*, 2009). Growth-inhibiting activity

against bacterial as well as fungal pathogens could indicate that **1** targets an essential cellular process that is conserved among both prokaryotic and eukaryotic microbes.

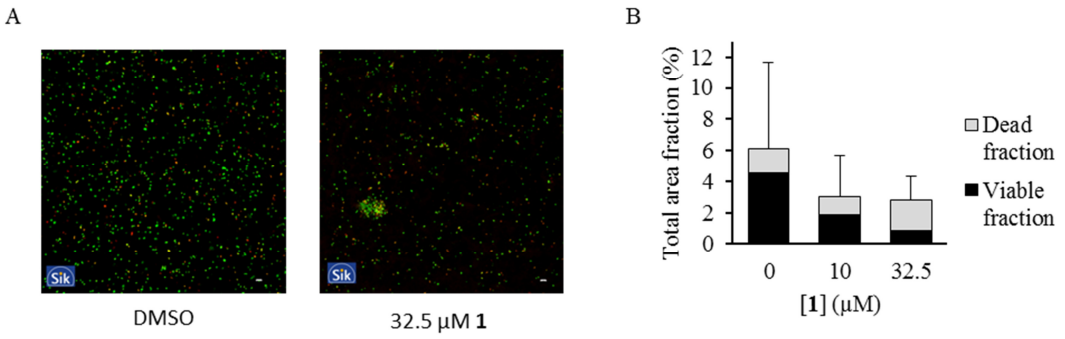


Figure I.3: CLSM analysis of biofilm inhibitory effect of 1. A. Representative CLSM micrographs of *P. aeruginosa* treated with **1**. The extent of the titanium surface that is covered by bacteria in a thin optical section at full width half maximum is shown after treatment with 0 and 32.5 µM of **1**. Live cells were stained with CYTO®9 (green) and dead cells were stained with propidium iodide (red). Scale bar = 5 µm. Each sample was analyzed in triplicate. **B.** Compound **1** reduces the total area fraction and proportion of viable cells of *P. aeruginosa* biofilms covering titanium discs. After 19 h of growth in the presence of compound **1** (0, 10 and 32.5 µM), the discs were analyzed with CLSM. The proportion of viable (black) and dead (light grey) cells is indicated in the graphs. This experiment was repeated independently 3 times, error bars represent standard error of the mean (SEM).

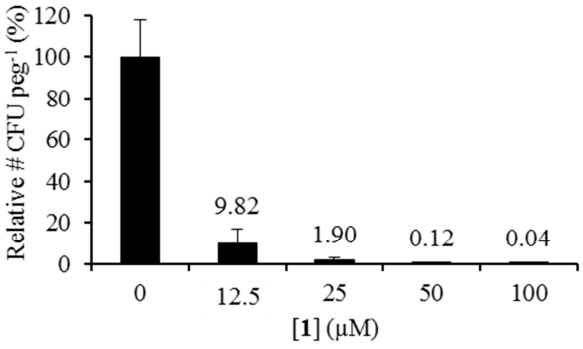


Figure I.4: Compound 1 **eradicates *P. aeruginosa* biofilms.** 24 h old biofilms of *P. aeruginosa* were subjected to a 24 h treatment with different concentrations of **1**. Subsequently, biofilm cells were disrupted by sonication, diluted and plated out after which the number of surviving cells was determined. This experiment was repeated independently three times, error bars represent SEM. The number of CFU peg^{-1} in the control sample was normalized to 100 % and corresponds to 4.62 x 10^5. The relative number of CFU peg^{-1} is indicated above each bar.

Table I.4: Minimal inhibitory concentration (MIC) and minimal biofilm inhibitory concentration (MBIC) of 1 against *P. aeruginosa*, *E. coli*, *S. aureus*, *S. epidermidis* and *P. gingivalis* strains

Strain	MIC 1 (µM)	MBIC 1 (µM)	Growth medium
P.aeruginosa			
PA14	50	50	1:20 TSB
YM	50	ND[a]	1:20 TSB
YM64	12.5	ND[a]	1:20 TSB
E. coli TG1	12.5	6[b]	1:20 TSB
S. aureus SH1000	25	25	MHB
S. epidermidis RP62A	25	25	MHB
P. gingivalis ATCC 33277	12.5	13.8[b]	1:20 TSB

[a]ND: not determined; [b]$MBIC_{90}$

I.4.4 Effect of 1 on cell viability

As limited human cellular toxicity is an important feature for a compound with such a broad spectrum of action, the toxicity of **1** was evaluated on several human cell types. While two hours of exposure to 12.5 µM of **1** resulted in > 80 % viability for osteoblasts and mesenchymal stem cells, these conditions were lethal to microvascular endothelial cells (data not shown). However, the tube formation potential of aortic endothelial cells was not negatively affected (Figure I.5), indicating that more differentiated endothelial cells in the tube-assembled stage are less susceptible to the potential toxic effects of **1** compared to endothelial cells in the non-assembled stage in monolayer culture. The observed difference could also be due to the use of different types of endothelial cells in the two assays, as endothelial cells from different sources are functionally different and could therefore behave differently. Moreover, various carbazoles have previously been shown to possess low cellular toxicity at 80-100 µM (Sunthitikawinsakul *et al.*, 2003), pointing to the possibility to further develop **1** into a non-toxic novel antipseudomonal biofilm drug and a broad-spectrum antibacterial and antifungal agent.

I.5 Conclusion

We identified **1**, a non-toxic N-alkylated 3, 6-dihalogenocarbazol displaying anti-pseudomonal activity both on planktonic and biofilm cultures. Furthermore**, 1** inhibits growth

of several other clinically important pathogens making **1** an interesting starting point for development of a broad-spectrum agent.

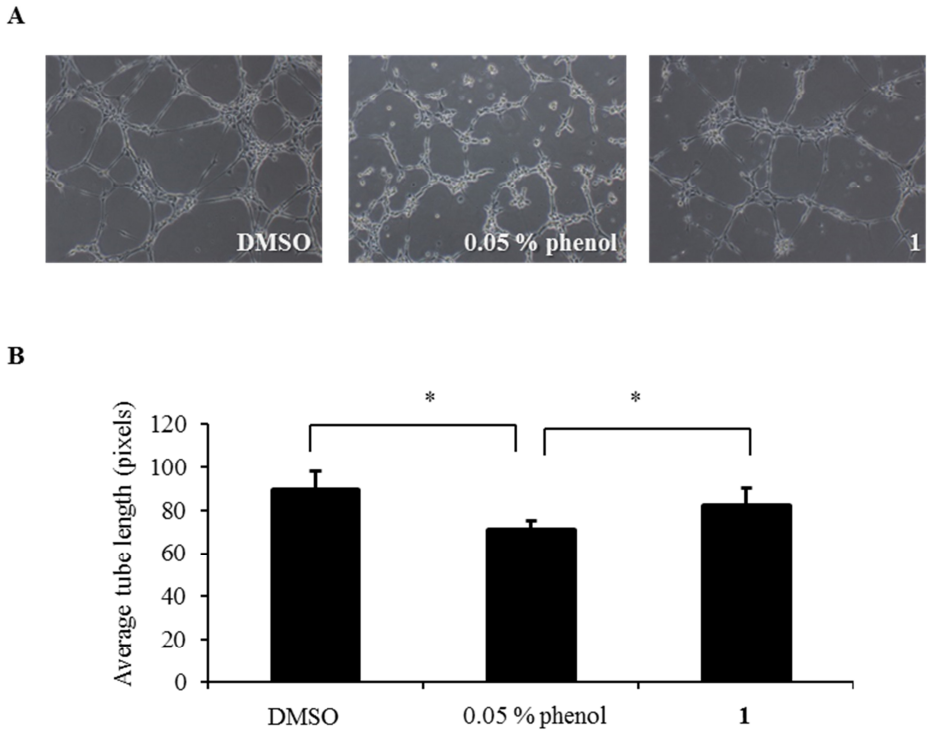


Figure I.5: Tube formation potential of endothelial cells is not affected by 1**.** Human endothelial cells were cultured in M200 medium with low serum growth supplement (ENDO) in the presence of 0.5 % dimethyl sulfoxide (DMSO) (control treatment), 0.05 % phenol (positive cytotoxic control) or 12.5 µM of **1** for 4 hours. **A**. Representative photographs and. **B.** average tube length are shown for each condition. The average tube length is not inhibited by **1**, whereas 0.05 % phenol causes negative effect on tube formation (* $P < 0.05$).

Appendix A

Optimization of DnpA overexpression for large-scale purification

A.1 Introduction

Based on its deduced amino acid sequence, DnpA is a member of the PIG-L or LmbE-like superfamily (Pfam PF02585). All eukaryotic members studied so far play a role in the synthesis of glycosylphosphatidylinositol, a glycolipid that anchors proteins at the cell surface (Nakamura *et al.*, 1997; Sharma *et al.*, 1997; Smith *et al.*, 1997; Watanabe *et al.*, 1999; Viars *et al.*, 2014). In bacteria, characterized PIG-L members are involved in different unrelated pathways as discussed in Chapter 4. These proteins share a catalytic site containing a number of conserved residues that are essential for enzymatic activity. Multiple sequence alignment of DnpA and all biochemically characterized members of this PIG-L family reveals that the critical residues of the active site are intact in DnpA. Therefore, we predict that DnpA acts as a de-*N*-acetylase, probably acting on *N*-acetylglucosamine residues of a yet unknown substrate. Evaluation of the persistence effect of mutant *dnpA* alleles, predicted to completely lack de-*N*-acetylase activity, led to contradictory results. Therefore, in order to answer the question if the predicted de-*N*-acetylase activity of DnpA is important in fluoroquinolone tolerance, data on biochemical activity are needed. For *in vitro* experiments, high amounts of purified DnpA are needed. In this Chapter, we describe the optimization of conditions for DnpA overexpression.

A.2 Experimental procedures

Strains and growth conditions. *Escherichia coli* strains were cultured in Luria-Bertani (LB) broth at 37 °C, for solidified medium 1.5 % agar was added. Following antibiotics were used:

kanamycin (45 µg mL^{-1}) and chloramphenicol (35 µg mL^{-1}). Strains and plasmids used in this study are listed in Table A.1.

Table A.1: Plasmids and strains used in this Chapter

Strain or plasmid	Description*	Reference/source
Strains		
E. coli TOP10	F-*mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) φ80*lac*ZΔM15 Δ*lac*X74 *deo*R *rec*A1 *ara*D139 Δ(*ara-leu*)7697 *gal*U *gal*K	Invitrogen
E. coli BL21–Codon Plus (DE3)-RP	F^- *ompT hsdS*(rB^- mB^-) *dcm*$^+$ Tet^R *gal* λ(DE3) *endA* Hte [*argU proL* Cm^R]	Stratagene
E. coli ROSETTA 2(DE3)pLysS	F^- *ompT hsdS*$_B$(r_B^- m_B^-) *gal dcm* (DE3) pLysSRARE2 (Cm^R)	Novagen
Plasmids		
pET24a(+)	pBR322, T7 promotor, Km^R, C-terminal His_6-tag	Novagen
pET28a(+)	pBR322, T7 promotor, Km^R, N-terminal His_6-tag	Novagen
pET24a(+)-*dnpA*-N	pET plasmid encoding N-terminal His_6-tagged DnpA	This work
pET24a(+)-*dnpA*-NT	pET plasmid encoding truncated (AA 1-43) N-terminal His_6-tagged DnpA	This work
pET28a(+)-*dnpA*-C	pET plasmid encoding C-terminal His_6-tagged DnpA	This work
pET28a(+)-*dnpA*-CT	pET plasmid encoding truncated (AA 1-43) C-terminal His_6-tagged DnpA	This work

*Tet^R: tetracycline resistance; Cm^R: chloramphenicol resistance; Km^R: kanamycin resistance

Construction of His_6-tagged DnpA. For optimization of expression, plasmids encoding different forms of His_6-tagged DnpA were constructed. For the construction of N-terminal His_6-tagged DnpA, amplification was carried out by using primer couples SPI5285-5286, SPI5285-5287 with purified genomic DNA as a template. The PCR fragments were XhoI-HindIII cloned into pET28a(+), in frame with the His_6-tag present in this plasmid.

Construction of C-terminal His_6-tagged DnpA was achieved by using primer couples SPI5367-5369 and SPI5368-5369. The resulting PCR fragments were cloned into pET24a(+) using XhoI-HindIII, in frame with the His_6-tag present on the plasmid. Primers SPI5367 and SPI5368 contain a start codon and a ribosome binding site (RBS). All constructs were confirmed by sequencing. Subsequently, the plasmids were introduced into *E. coli* BL21–Codon Plus (DE3)-RP by means of transformation. For sequences of the primers see Table A.2.

Table A.2: Primers used in this Chapter

Primer name	Sequence*	Description
SPI5285	CACC**AAGCTT**GCGCACGCAAGCAG	Reverse primer for construction of N-terminal His_6-tagged *dnpA*
SPI5286	ACTC**CTCGAG**TCACGCCTCCCCAT	Forward primer for construction of full length N-terminal His_6-tagged *dnpA*
SPI5288	CACC**AAGCTT**TGGCCCTGTGGGTC	Reverse primer for construction of truncated N-terminal His_6-tagged *dnpA*
SPI5367	ACTC**AAGCTT**AATAATTTTGTTTAA CTTTAAGAAGGAGATATACATATG AGCGCACGCAAGCAGCAGTTGCTC AAGCGTCATCGACGC	Reverse primer for construction of full length C-terminal His_6-tagged *dnpA*. Contains RBS and a start codon.
SPI5368	ACTC**AAGCTT**AATAATTTTGTTTAA CTTTAAGAAGGAGATATACATATG GTGGCCCTGTGGGTCGCCCACGAA GCC	Reverse primer for construction of truncated C-terminal His_6-tagged *dnpA*. Contains RBS and a start codon.
SPI5369	CACC**CTCGAG**CGCCTCCCCATCCA GCGACTC	Forward primer for construction of C-terminal His_6-tagged *dnpA*

*Restriction sites are indicated in bold; the start codon is underlined

Purification of total protein fraction on small scale. Overnight cultures were diluted 100-fold into LB medium and incubated at 37 °C while shaking at 200 rpm. After three hours of growth, cultures were induced with 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) for the indicated time after which 1 mL of culture was harvested by centrifugation (13 000 rpm, 1 minute, 4 °C). The resulting cell pellet was stored overnight at – 80 °C. The next day, the

pellet was resuspended in native lysis buffer (10 mM imidazole, 50 mM NaH_2PO_4, 300 mM NaCl, pH 8.0) and treated with lysozyme for 30 minutes. Soluble proteins were separated from the cell debris by centrifugation (13 000 rpm, 10 minutes, 4 °C). The remaining pellet was resuspended in an isovolume of denaturating lysis buffer (100 mM NaH_2PO_4, 10 mM Tris/HCl, 8 M Urea, pH 8.0). The presence of DnpA-His_6 in each fraction was analyzed by western hybridization.

Purification of total protein fraction on large scale. Overnight cultures were diluted 100-fold into LB medium and incubated at 37 °C while shaking at 200 rpm. After three hours of growth, cultures were induced with 1 mM IPTG overnight at 22 °C. Thereafter, the cells were harvested by centrifugation (4750 rpm, 20 minutes, 4 °C). The resulting cell pellet was stored overnight at – 80 °C. Next, the pellet was resuspended in native lysis buffer and treated with lysozyme (Sigma) for 30 minutes at 37 °C**.** To increase yields, cells were further disrupted by means of sonication. Next, samples were treated with Benzonase Nuclease (Sigma) in order to digest nucleic acids. Finally, proteins were separated from cell debris by centrifugation (9250 rpm, 25 minutes, 4 °C). The presence of DnpA-His_6 was verified by western hybridization.

Sodium dodecyl sulphate polyacrylamide gel electrophoresis analysis (SDS-PAGE) analysis. An aliquot of each protein sample was mixed with NuPAGE LDS Sample Buffer and Reducing Agent and incubated at 70 °C for 10 minutes. The samples were loaded onto a NuPAGE Novex 12 % Bis-Tris MiniGel (Invitrogen), using a MOPS-based running buffer (50 mM MOPS, 50 mM Tris, 1 mM EDTA, 0.1 % SDS (w/v), pH 7.7). Visualization of the proteins was carried out by incubating the protein gel in a Coomassie blue solution for 30 minutes, followed by a destaining procedure (destaining solution: 45 % methanol (v/v), 7 % acetic acid (v/v)) until the desired background was reached.

Western blotting and hybridization. Proteins were transferred from a NuPAGE Novex 12 % Bis-Tris MiniGel (Invitrogen) to a polyvinylidene fluoride membrane using an XCell II Blot Module (Invitrogen) using CAPS buffer (10 mM CAPS, 10 % methanol (v/v), pH 11.0) as a transfer buffer. Successful transfer was verified by Coomassie blue staining of the protein gel. Western hybridization was performed using a monoclonal anti-His_6 antibody solution (Roche) and Anti-Mouse IgG Alkaline Phosphatase Conjugated antibodies (Sigma) as a primary and secondary antibody, respectively.

A.3 Results

A.3.1 Optimization of DnpA overexpression using *E. coli* BL21–Codon Plus (DE3)-RP as a host strain

To optimize overexpression of DnpA, small-scale experiments were carried out. DnpA is predicted to possess a single transmembrane helix which anchors the protein into the bacterial inner membrane (see Chapter 5). Therefore, in addition to the full length allele, a truncated version which lacks the first 43 N-terminal amino acids was constructed, including part of the predicted transmembrane domain. For each of these alleles, two different forms were constructed. One of them contains an N-terminal His_6-tag, the other one is C-terminally tagged with His_6. For these experiments, *E. coli* BL21–Codon Plus (DE3)-RP was used as a host strain. This strain contains extra copies of genes encoding tRNAs that recognize the arginine codons AGA and AGG and the proline codon CCC, respectively. Translation of heterologous proteins from GC-rich genomes such as *Pseudomonas aeruginosa* is most frequently restricted by these tRNAs.[22]

Overnight cultures were diluted 100-fold into LB medium and incubated at 37 °C while shaking at 200 rpm. After three hours, cells were induced with 1 mM IPTG during 1, 2, 3 and 4 hours at 22 °C. Next, cells were harvested by centrifugation after which the total protein fraction was purified as described in Experimental procedures. As shown in Figure A.1 most His_6-tagged protein was detected in the soluble fraction when overexpressing the allele encoding full length DnpA containing a C-terminal His_6-tag. Therefore, further optimization was carried out using the pET28a(+)-*dnpA*-C construct.

To increase the amount of DnpA in the soluble fraction, induction at 16 °C was tested. In addition, phenylmethylsulfonyl fluoride was added to all lysis buffers to prevent protein degradation by proteases. As a control, an *E. coli* strain overexpressing the *obgE-venus*-His_6 allele was taken along in these experiments. ObgE-Venus-His_6 is known to occur both in the soluble and insoluble fraction, but sufficient amount of the protein is present in the soluble fraction to ensure successful purification (Natalie Verstraeten, personal communication). Overnight cultures were diluted 100-fold into LB medium and incubated at 37 °C while shaking at 200 rpm. After three hours, cells were induced with 1 mM IPTG during 4, 8, 52

[22] www.genomics.agilent.com/article.jsp?pageId=484

and 68 hour at 16 °C. Next, cells were harvested by centrifugation after which the total protein fraction was purified as described in Experimental procedures. Equal volumes of all protein samples were loaded on the protein gel. As shown in Figure A.2, DnpA is mostly present in the insoluble fraction.

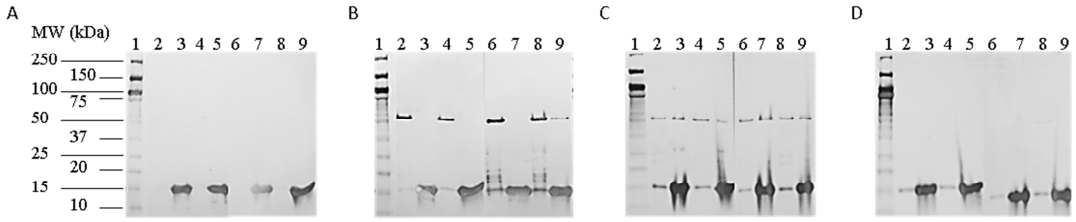


Figure A.1: Detection of DnpA-His$_6$ after overexpression in *E. coli* BL21–Codon Plus (DE3)-RP cells. Western hybridization analysis of soluble and insoluble total protein fractions after 1, 2, 3 and 4 hours of induction of *E. coli* BL21–Codon Plus (DE3)-RP cells overexpressing **A.** full length N-terminal His$_6$-tagged DnpA (theoretical MW of 57.67 kDa) **B.** truncated N-terminal His$_6$-tagged DnpA (theoretical MW of 52.94 kDa) **C.** full length C-terminal His$_6$-tagged DnpA (theoretical MW of 54.51 kDa) and **D.** truncated C-terminal His$_6$-tagged DnpA (theoretical MW of 49.78 kDa). Equal volumes of each fraction were evaluated. Analysis was carried out with an anti-His$_6$ primary antibody. Lane 1: Precision Plus Protein Kaleidoscope Standards (Bio-Rad); lane 2, 4, 6 and 8: insoluble protein fraction; lane 3, 5, 7 and 9: soluble protein fraction after 1, 2, 3 and 4 hours of induction respectively. For clarity, the order of the lanes of protein gels B and C was switched.

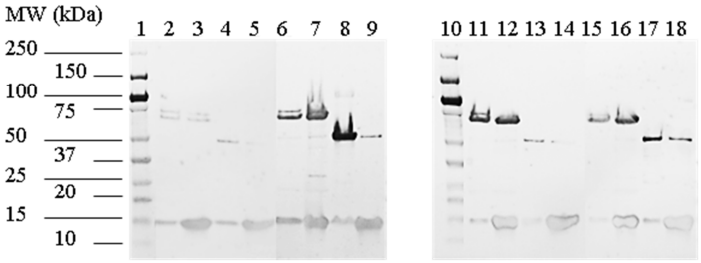


Figure A.2: Detection of DnpA-His$_6$ and ObgE-Venus-His$_6$ after overexpression in *E. coli* BL21–Codon Plus (DE3)-RP cells. Western hybridization analysis of soluble and insoluble total protein fractions after 4, 28, 52 and 68 hours of induction of *E. coli* BL21–Codon Plus (DE3)-RP cells overexpressing full length C-terminal His$_6$-tagged DnpA (theoretical MW of 54.51 kDa) or ObgE-Venus-His$_6$ (theoretical MW of 75.48 kDa). Equal volumes of each fraction were evaluated. Analysis was carried out with an anti-His$_6$ primary antibody. Lane 1 & 10: Precision Plus Protein Kaleidoscope Standards (Bio-Rad); lane 2, 6, 11 and 15 represent insoluble protein fractions; lane 3,7, 12 and 16 represent soluble protein fractions after 4, 28, 52 and 68 hours induction of full length ObgE-Venus-His$_6$ respectively; lane 4, 8, 13 and 17 represent insoluble protein fractions; lane 5,9, 14 and 18 represent soluble protein fractions after 4, 28, 52 and 68 hours induction of full length C-terminal His$_6$-tagged DnpA respectively. For clarity, the order of the lanes of the first protein gel was switched.

A.3.2 Optimization of DnpA overexpression using *E. coli* ROSETTA 2(DE3) pLysS as a host strain

When checking the *dnpA* nucleotide sequence for rare codons[23] (Clarke & Clark, 2008), it was observed that 1 – 7 % of the gene contains uncommon codons, which could significantly influence expression. Therefore, expression of *dnpA* was checked in an *E. coli* ROSETTA 2 (DE3) pLysS host strain. This strain contains tRNAs for seven rare codons (AGA, AGG, AUA, CUA, GGA, CCC, and CGG), encoded on a compatible chloramphenicol-resistant plasmid. In addition, this strain harbors the plasmid pLysS which expresses T7 lysozyme. This enzyme further suppresses basal expression of T7 RNA polymerase prior to induction. In this way, cells that harbor plasmids encoding a protein that affects cell growth and viability are stabilized.[24] Additionally, since *dnpA* encodes a hypothetical inner membrane protein (see Chapter 5), overexpression of DnpA possibly destabilizes the inner membrane of the cell. Therefore, a very short induction time of 0.5 hours was tested.

Overnight cultures were diluted 100-fold into LB medium and incubated at 37 °C while shaking at 200 rpm. After three hours, cells were induced with 1 mM IPTG during 0.5, 1, 4, 20 and 28 hours at 16 °C. Total protein fraction was purified as described in Experimental procedures. DnpA-His_6 was not detected after 0.5 hour of induction, indicating that incorporation of DnpA-His_6 in the membrane does not significantly affect membrane stability. An induction time of 20 hours resulted in the highest amount of DnpA present in the soluble fraction (Figure A.3A).

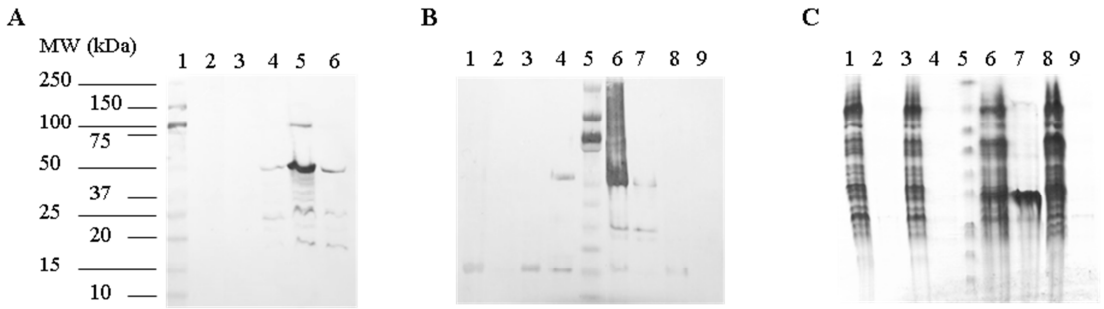
The three other constructs containing the truncated C- and N-terminal His_6-tagged *dnpA* alleles (encoding DnpA-CT and DnpA-NT respectively) and the full length N-terminal His_6-tagged *dnpA* allele (encoding DnpA-N), were tested for expression under these optimized conditions. The corresponding plasmids were introduced into the *E. coli* ROSETTA 2(DE3) pLysS host strain by transformation. Next, expression was evaluated after 20 hours of induction at 22 °C. In addition, to evaluate if DnpA-His_6 can be purified from the total soluble protein fraction by its His_6-tag, the "QuickPick™ IMAC" Kit (Bio-Nobile Oy) was used. This technique allows purification of His_6-tagged proteins on a small scale by means of metal affinity. The results, presented in Figure A.3B, show that only overexpression of the allele

[23] www.codons.org and www.genscript.com

[24] www.emdmillipore.com

encoding full length C-terminal His_6-tagged DnpA results in detectable soluble protein. Overexpression of the other *dnpA* alleles did not yield detectable His_6-tagged protein in any of the protein fractions, with the exception of a low amount of DnpA-NT present in the insoluble fraction. Furthermore, Figure A.3C shows that the His_6-tag of DnpA-C-His_6 is accessible, allowing purification by means of metal affinity.

These results suggest that the predicted transmembrane helix does not cause any problems during overexpression of *dnpA*. This is in accordance with earlier published data on the PIG-L protein from *Rattus norvegicus*. This protein, which also contains a transmembrane domain, was proven to be functional upon overexpression and purification using *E. coli* host cells (Watanabe *et al.*, 1999).



Figue A.3: Evaluation of overexpression *dnpA* in *E. coli* ROSETTA 2(DE3)pLysS cells. A. Western hybridization analysis of soluble total protein fractions after 0.5, 1, 4, 20 and 28 hours of induction of *E. coli* ROSETTA 2(DE3)pLysS cells overexpressing full length C-terminal His_6-tagged DnpA (theoretical MW of 54.51 kDa). Equal volumes of each sample were analyzed. Lane 1: Precision Plus Protein Kaleidoscope Standards (Bio-Rad); lane 2-6 represent soluble protein fractions after 0.5, 1, 4, 20 and 28 hours induction of full length C-terminal His_6-tagged DnpA, respectively. **B.** Western hybridization analysis of soluble and insoluble total protein fractions after 20 hours of induction of *E. coli* ROSETTA 2(DE3)pLysS cells overexpressing full length N-terminal His_6-tagged DnpA (DnpA-N), truncated N-terminal His_6-tagged DnpA (DnpA-NT), full length C-terminal His_6-tagged DnpA (DnpA-C, theoretical MW of 54.51 kDa) and truncated C-terminal His_6-tagged DnpA (DnpA-CT). Equal volumes of each fraction were analyzed. Analysis was carried out with an anti-His_6 primary antibody. Lane 5: Precision Plus Protein Kaleidoscope Standards (Bio-Rad); lane 1, 3, 6 and 8 represent soluble protein fractions; lane 2, 4, 7 and 9 represent insoluble fractions after overexpression of *dnpA*-N, *dnpA*-NT, *dnpA*-C and *dnpA*-CT respectively. **C.** Coomassie blue staining of total soluble protein fractions (lane 1, 3, 6 and 8) and proteins purified by metal affinity chromatography (lane 2, 4, 7 and 9) separated by SDS-PAGE after overexpression of *dnpA*-N, *dnpA*-NT, *dnpA*-C and *dnpA*-CT respectively. Lane 5: Precision Plus Protein Kaleidoscope Standards (Bio-Rad).

A.3.3 Large-scale purification of DnpA-His_6

Next, full length C-terminal His_6-tagged DnpA was purified on large scale by using high pressure liquid chromatography (HPLC) affinity chromatography using an ÄKTApurifier (GE healthcare). Total protein fractions were isolated as described in Experimental procedures and loaded onto a HisTrap HP column, washed (wash buffer: 20 mM imidazole, 50 mM NaH_2PO_4, 300 mM NaCl, pH 8.0) and bound recombinant proteins were eluted by competition with imidazole (elution buffer: 250 mM imidazole, 50 mM NaH_2PO_4, 300 mM NaCl, pH 8.0). Further purification was carried out by gel filtration using a HiLoad 16/60 Superdex 200 pg column (GE Healthcare) in a 50 mM NaCl 20 mM Tris buffer (pH 7.2). Despite the presence of DnpA-His_6 in the soluble protein fraction, it was not possible to retain the protein by size exclusion chromatography. Therefore, protein fractions belonging to each independent step throughout the purification process were evaluated for the presence of DnpA-His_6 by western hybridization (Figure A.4). The result shows that DnpA-His_6 is present in the soluble protein fraction but elutes with the void volume during gel filtration. In addition, there was significant loss of DnpA-His_6 during the different washing steps. To optimize the purification process, a collaboration was started with Prof. Wim Versées (Structural Biology Brussels, Vrije Universiteit Brussels, Belgium; Department of Molecular and Cellular Interactions, VIB, Belgium). However, no conditions were found that allowed successful purification of DnpA-His_6.

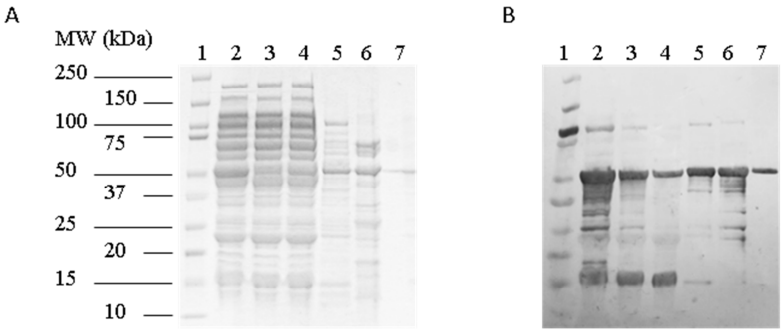


Figure A.4: A. Coomassie blue stained protein gel and **B.** western hybridization of the different protein fractions during the isolation and purification process of DnpA-His_6 by means of Ni^{2+}-chelation HPLC affinity chromatography followed by size exclusion chromatography (SEC). Lane 1: Precision Plus Protein Kaleidoscope Standards (Bio-Rad); Lane 2-7: total soluble protein fraction, void volume, washing step 1, washing step 2, eluate and void volume of SEC, respectively.

A.3.4 Optimization of DnpA overexpression using additional truncated constructs

Meanwhile, the topology of DnpA was determined (see Chapter 5). Based on these results, additional alleles encoding His_6-tagged DnpA lacking the first 60 and 178 amino acids, respectively, were tested. These truncated *dnpA* alleles were constructed and evaluated by the research group of Prof. Wim Versées (Structural Biology Brussels, Vrije Universiteit Brussels, Belgium; Department of Molecular and Cellular Interactions, VIB, Belgium). No expression was observed using the plasmid encoding DnpA-N178, while low expression was obtained with the plasmid encoding DnpAN60 (data not shown) (Wim Versées, personal communication).

A.4 Conclusion

In this Chapter, we optimized conditions for DnpA overexpression using different host strains and *dnpA* alleles. Good expression was obtained with the full length C-terminal His_6-tagged DnpA. However, during large scale purification, the protein eluted with the void volume during gel filtration. Several other forms of DnpA, lacking one or two transmembrane segments, were constructed and tested. One of those forms, DnpAN60, showed promising results although further optimization is needed to increase expression levels.

Appendix B

Additional Tables and Figures

Table B.1: Effect of compounds that in combination with ofloxacin increase the number of surviving cells. The number of cells upon treatment was determined based on the correlation between the number of cells and the duration of the lag phase after dilution of the treated cells into fresh growth medium. The relative fold increase in surviving cells was calculated as described in Chapter 6. The experiment was repeated independently three times.

Plate	Well	Relative fold increase in surviving cells	SEM*
208	C2	263.82	23.52
182	D10	4.40	0.54
171	F5	5.57	0.40
141	G10	665.96	263.56
84	F3	89.98	21.14
81	H2	60.10	19.41
81	F6	15.78	3.28
80	G1	83.25	8.09
80	B1	14.50	3.93
79	C2	15.70	11.36
75	B4	500.11	60.17
70	A2	2.52	0.51
65	F10	11.09	1.22
56	D2	11.16	2.91
56	E2	5.61	0.53
55	E6	83.14	15.54
51	G2	10.11	3.06
50	A10	3.56	0.70
50	D10	3.54	0.85
15	D7	6.16	0.45
13	A3	4.45	1.00
4	H6	134.75	21.98
4	F10	113.36	11.24
3	D5	11.81	1.33

*SEM, standard error of the mean

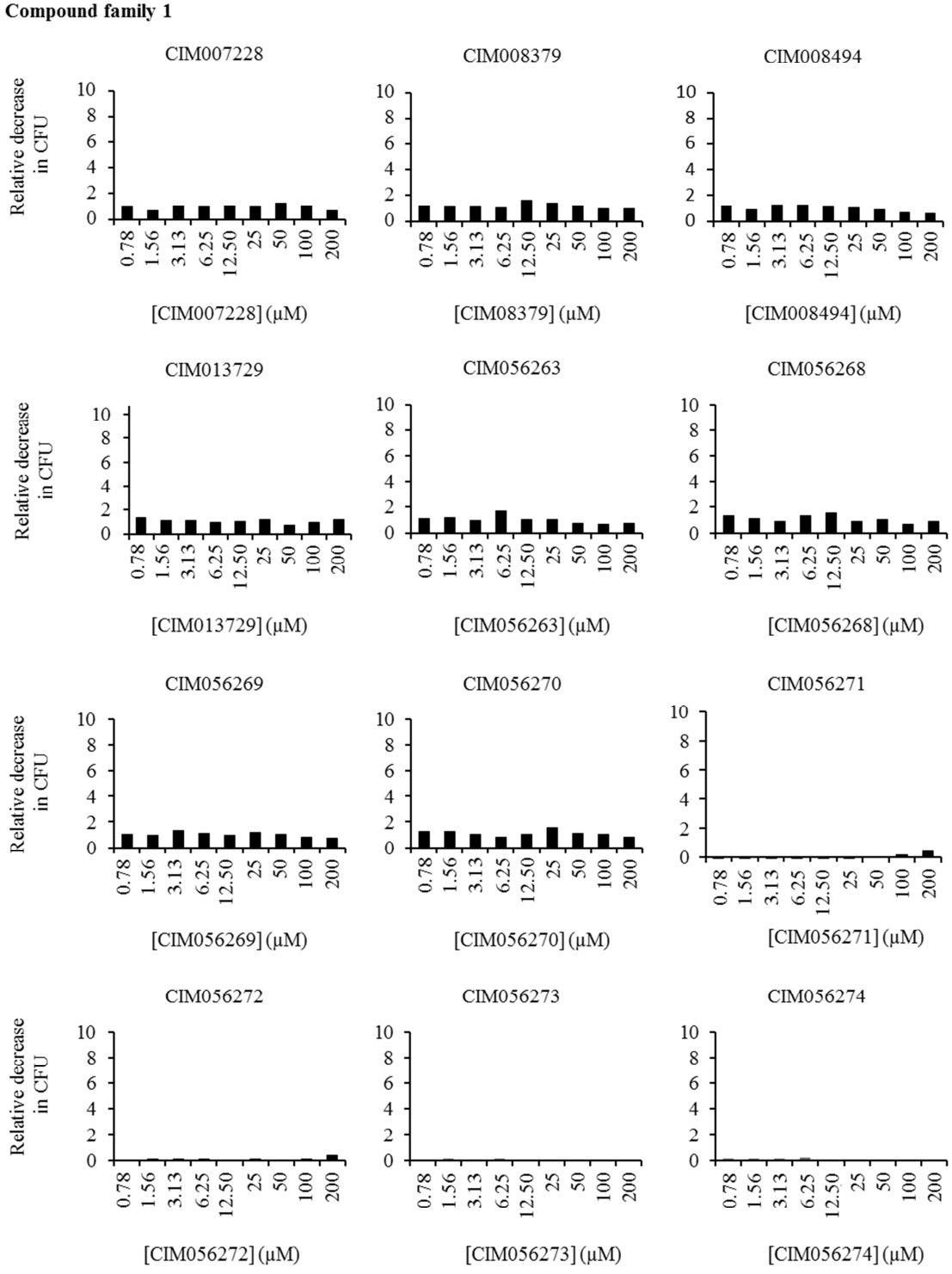
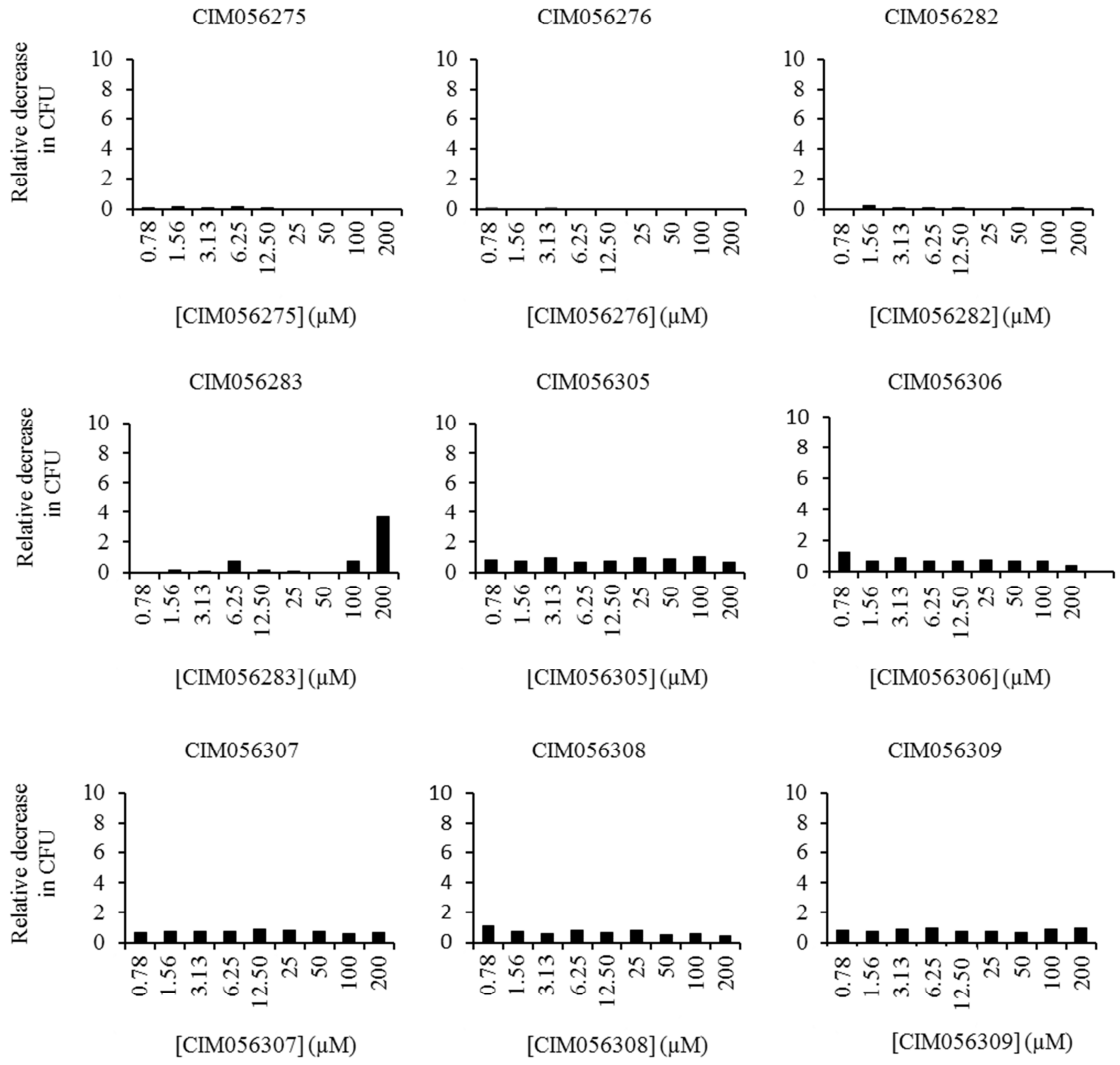
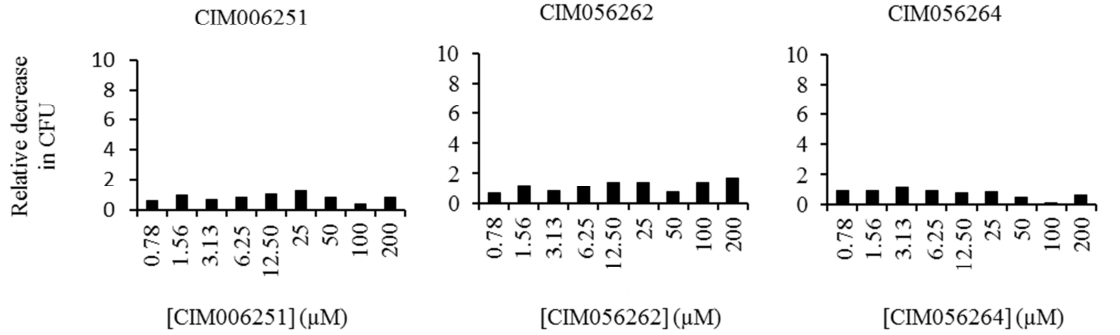


Figure B.1: Bactericidal effect of chemical analogues (continued).

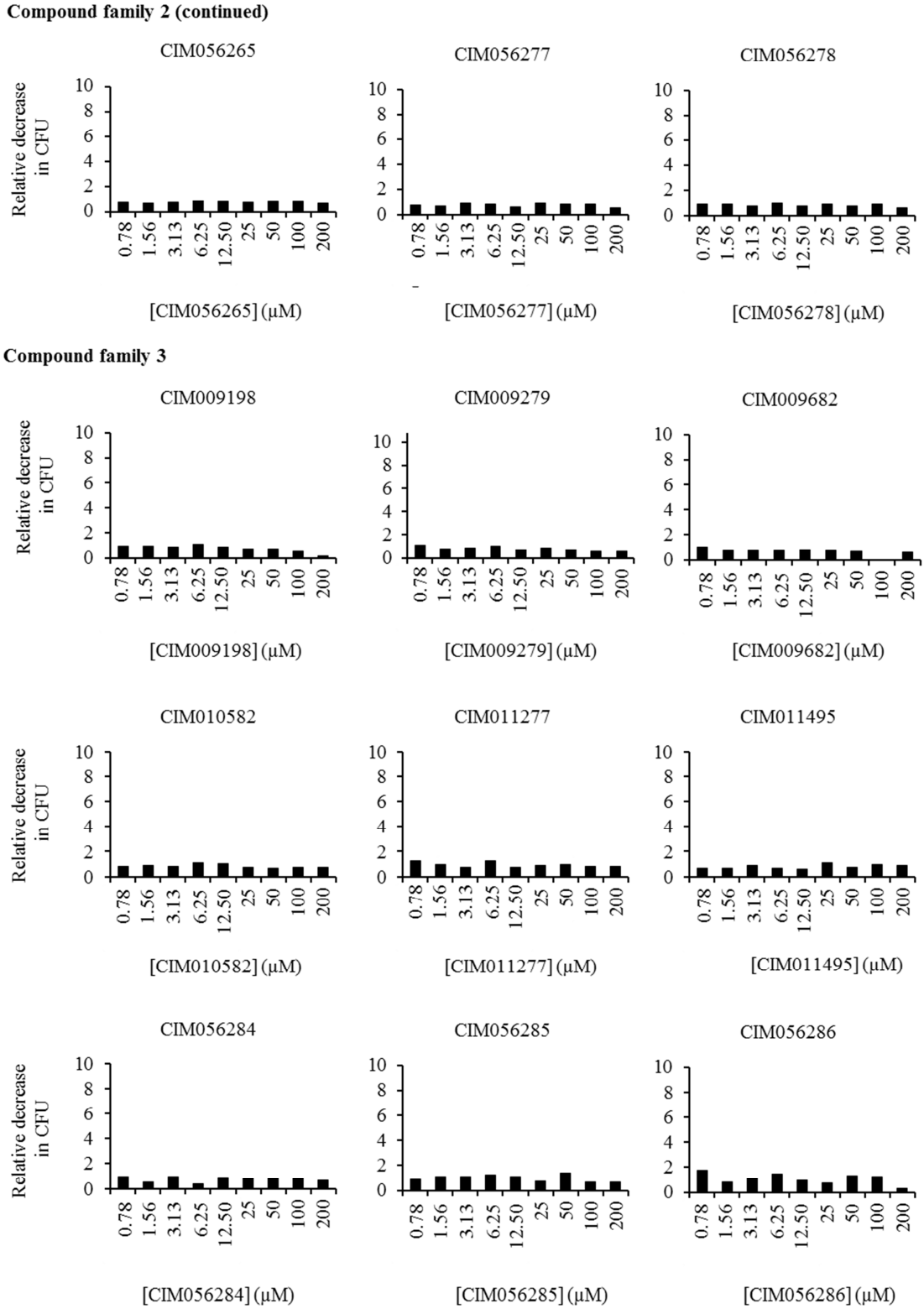
Compound family 1 (continued)



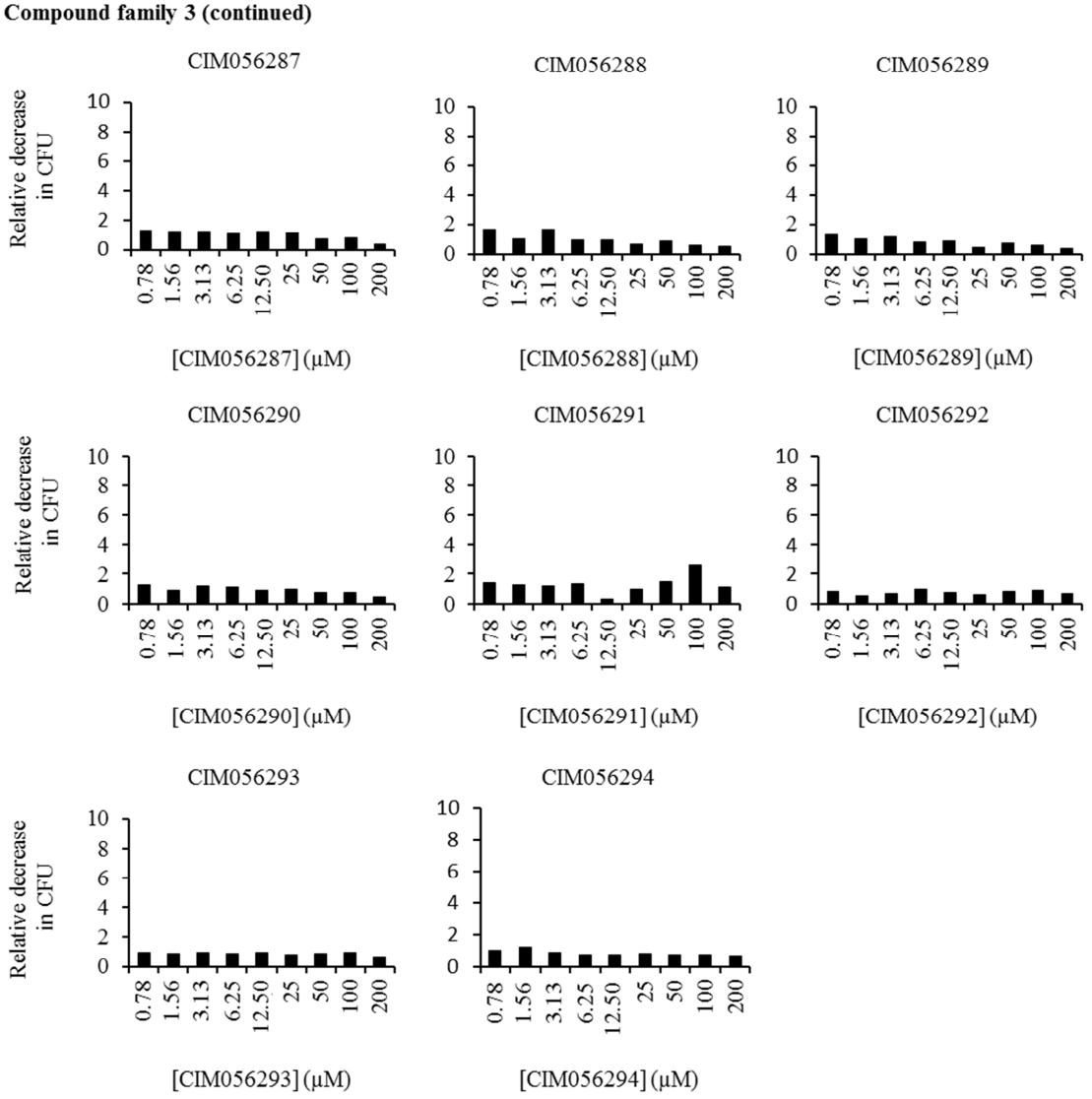
Compound family 2



Continued Figure B.1: Bactericidal effect of chemical analogues (continued).



Continued Figure B.1: Bactericidal effect of chemical analogues (continued).



Continued Figure B.1: Bactericidal effect of chemical analogues. Stationary phase cells were treated for 5 hours with compound (0.78 – 200 µM). After treatment, samples were 100-fold diluted into fresh Trypticase Soy Broth (TSB) growth medium and incubated in an automated plate reader in order to obtain separate growth curves for each sample. Based on the duration of the lag phase of each individual growth curve, the number of surviving cells was estimated. Data points represent the relative reduction in number of cells compared to treatment with only DMSO.

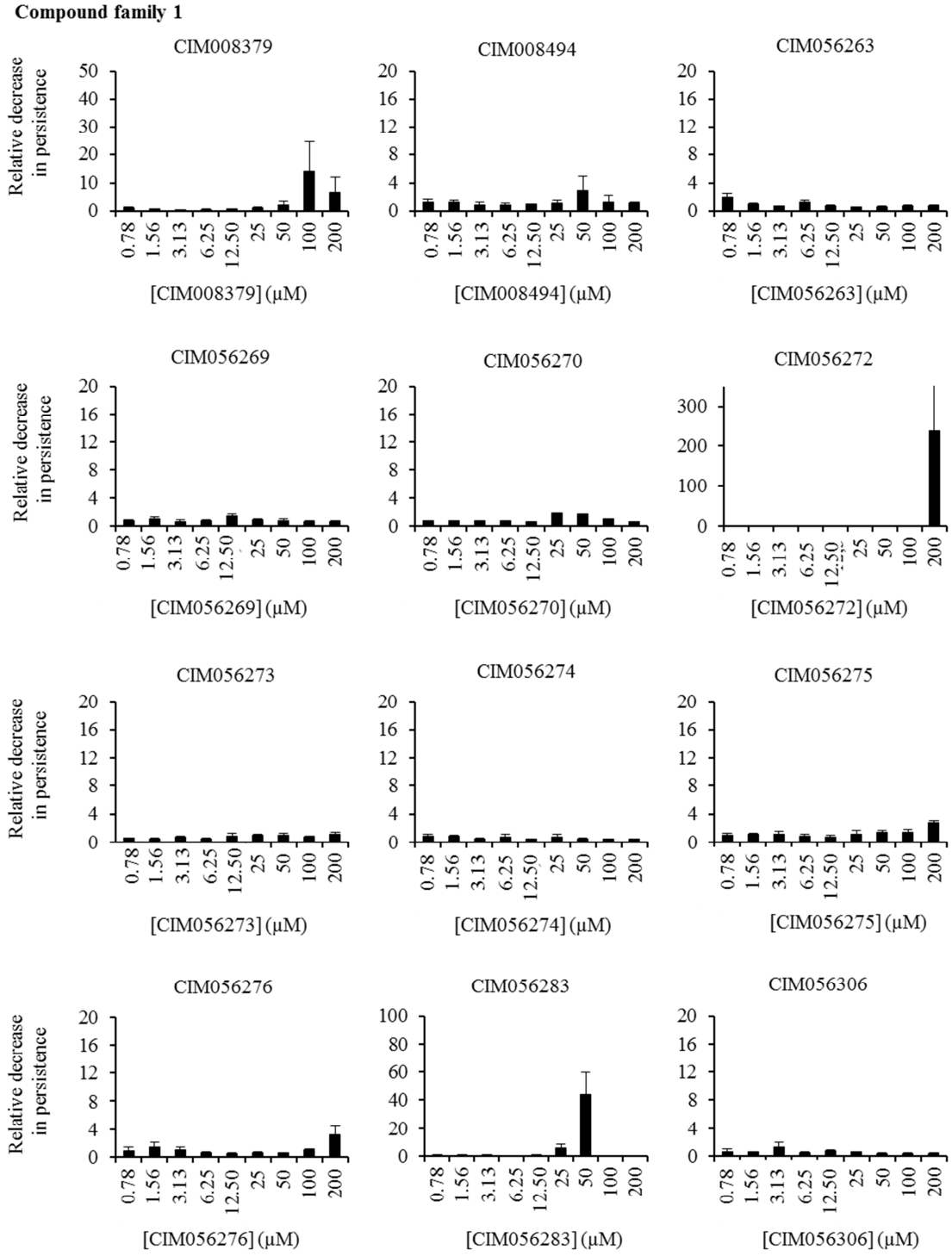
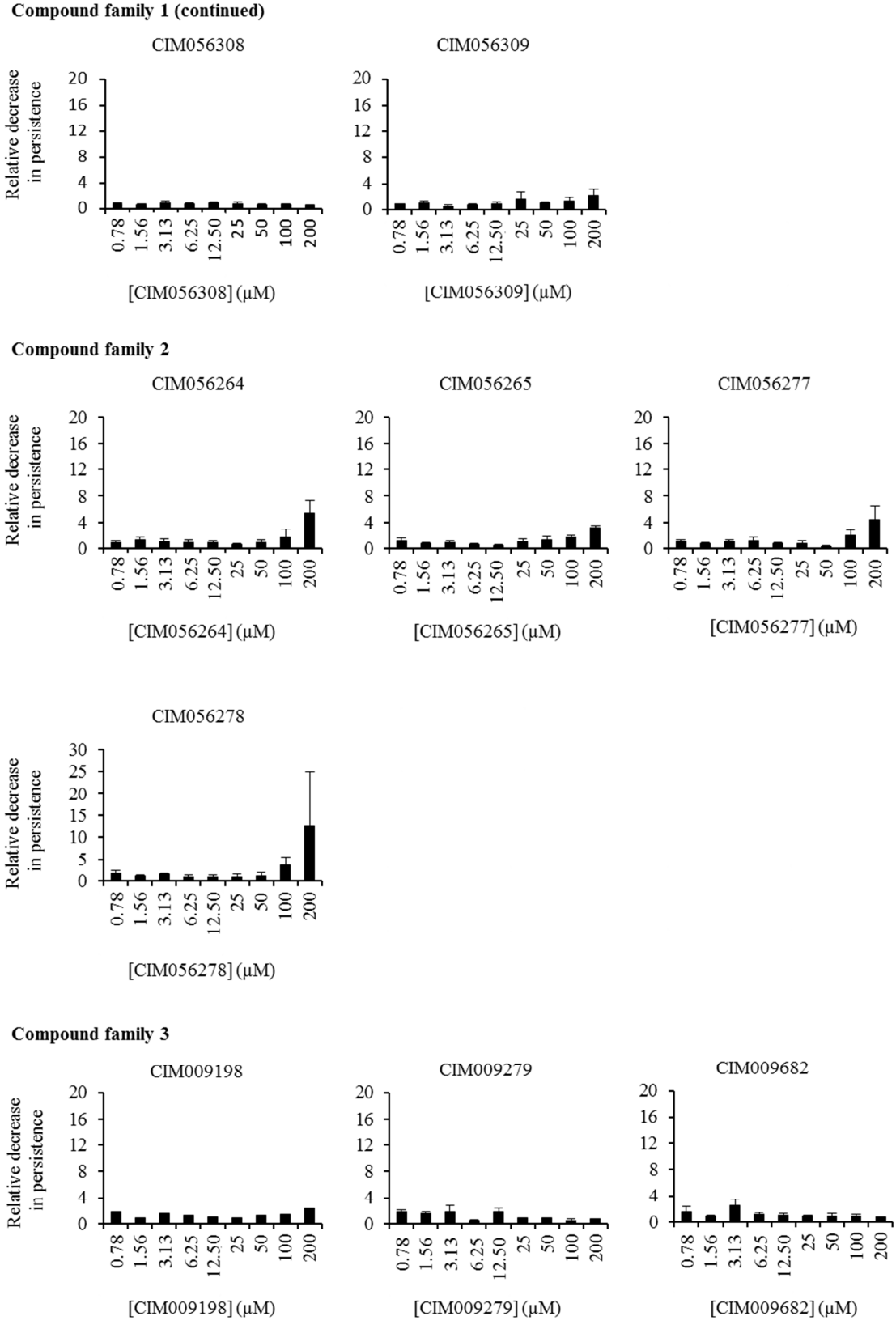
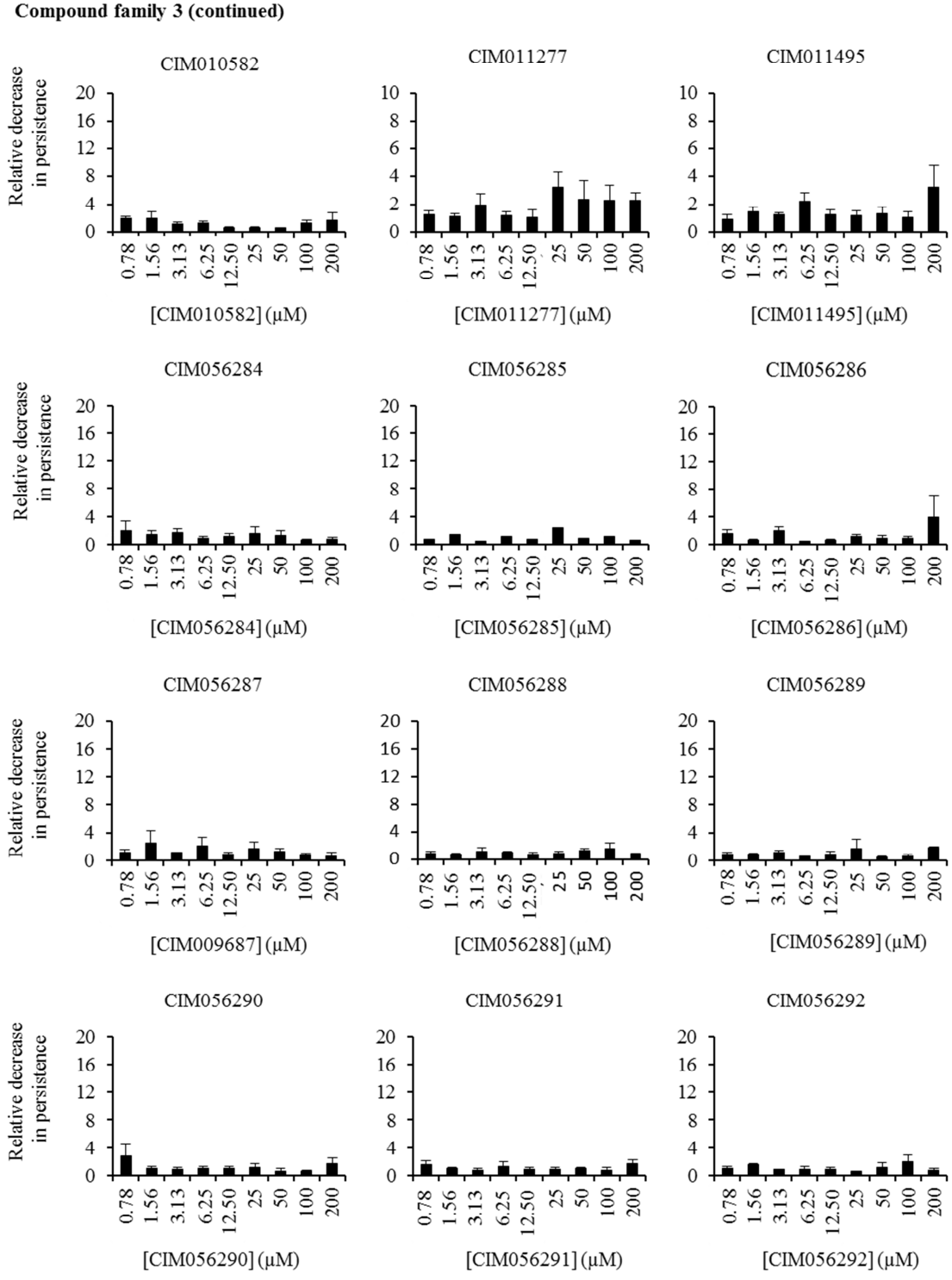


Figure B.2: Effect on persistence of all chemical analogues (continued)

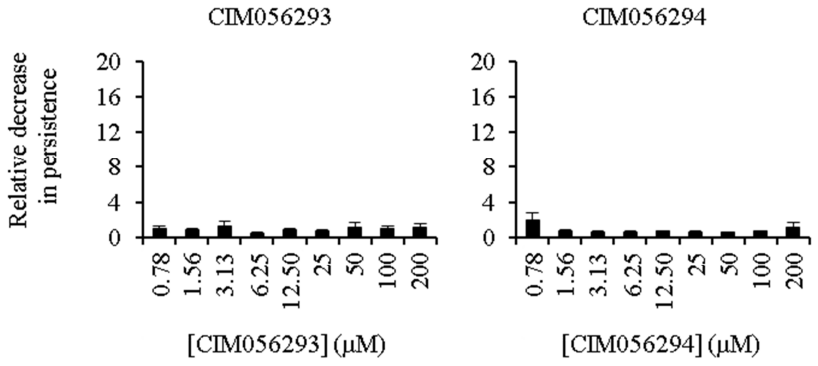


Continued Figure B.2: Effect on persistence of all chemical analogues (continued).



Continued Figure B.2: Effect on persistence of all chemical analogues (continued).

Compound family 3 (continued)



Continued Figure B.2: Effect on persistence of all chemical analogues in combination with ofloxacin. Stationary phase cells were treated for 5 hours with a combination of ofloxacin (10 µg mL-1) and compound (0.78 – 200 µM). After treatment, samples were 100-fold diluted into fresh TSB growth medium and incubated in an automated plate reader in order to obtain separate growth curves for each sample. Based on the duration of the lag phase of each individual growth curve, the number of surviving cells was estimated. Data points represent the relative reduction in number of cells compared to treatment with only ofloxacin.

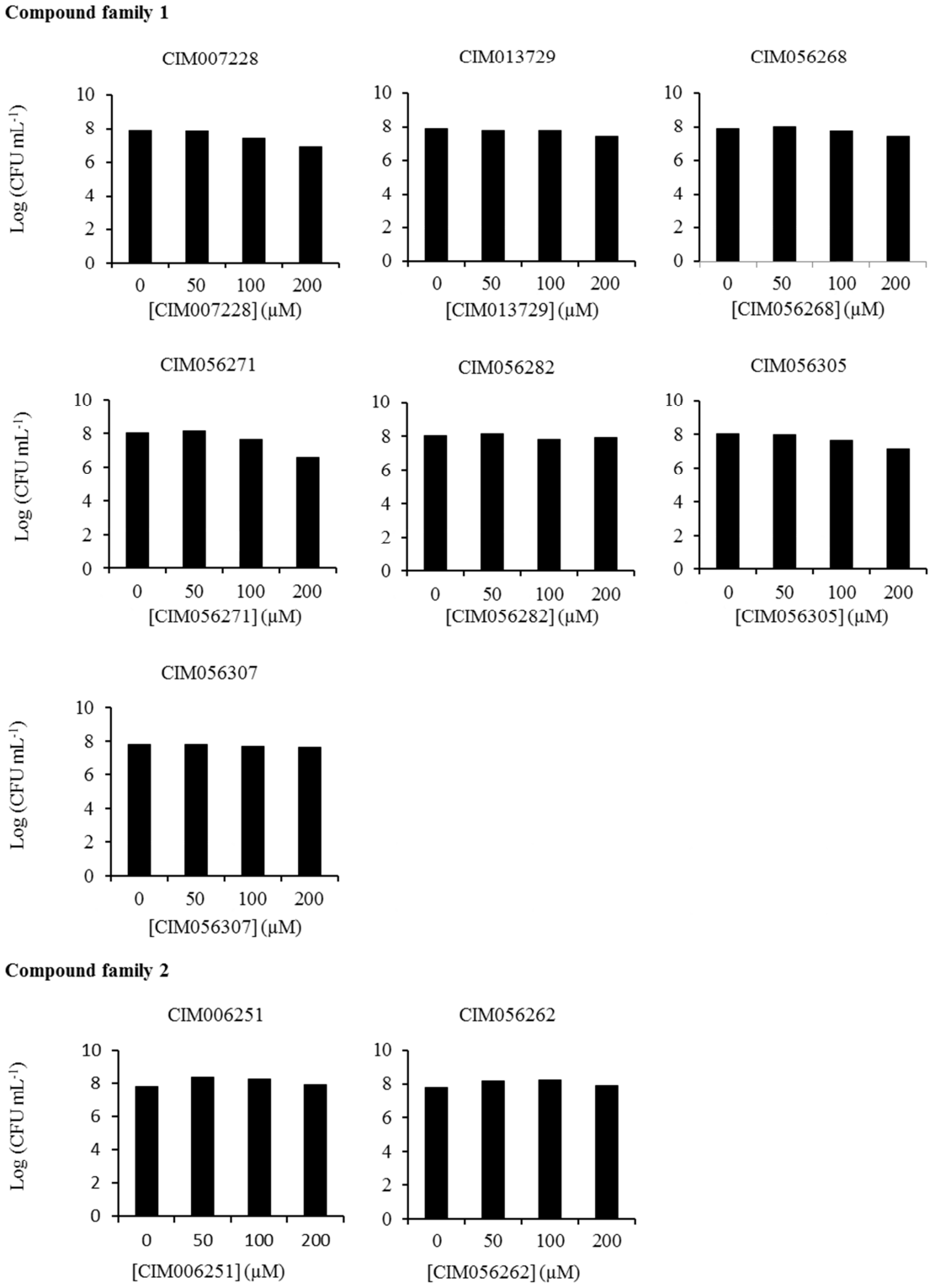


Figure B.3: Bactericidal effect of selected analogues on stationary phase cultures. Stationary phase cells were treated for 5 hours with compound (0 – 200 µM). After treatment, samples were diluted and plated on solid growth medium in order to determine the number of viable cells. Data points represent the relative decrease in viable cells.

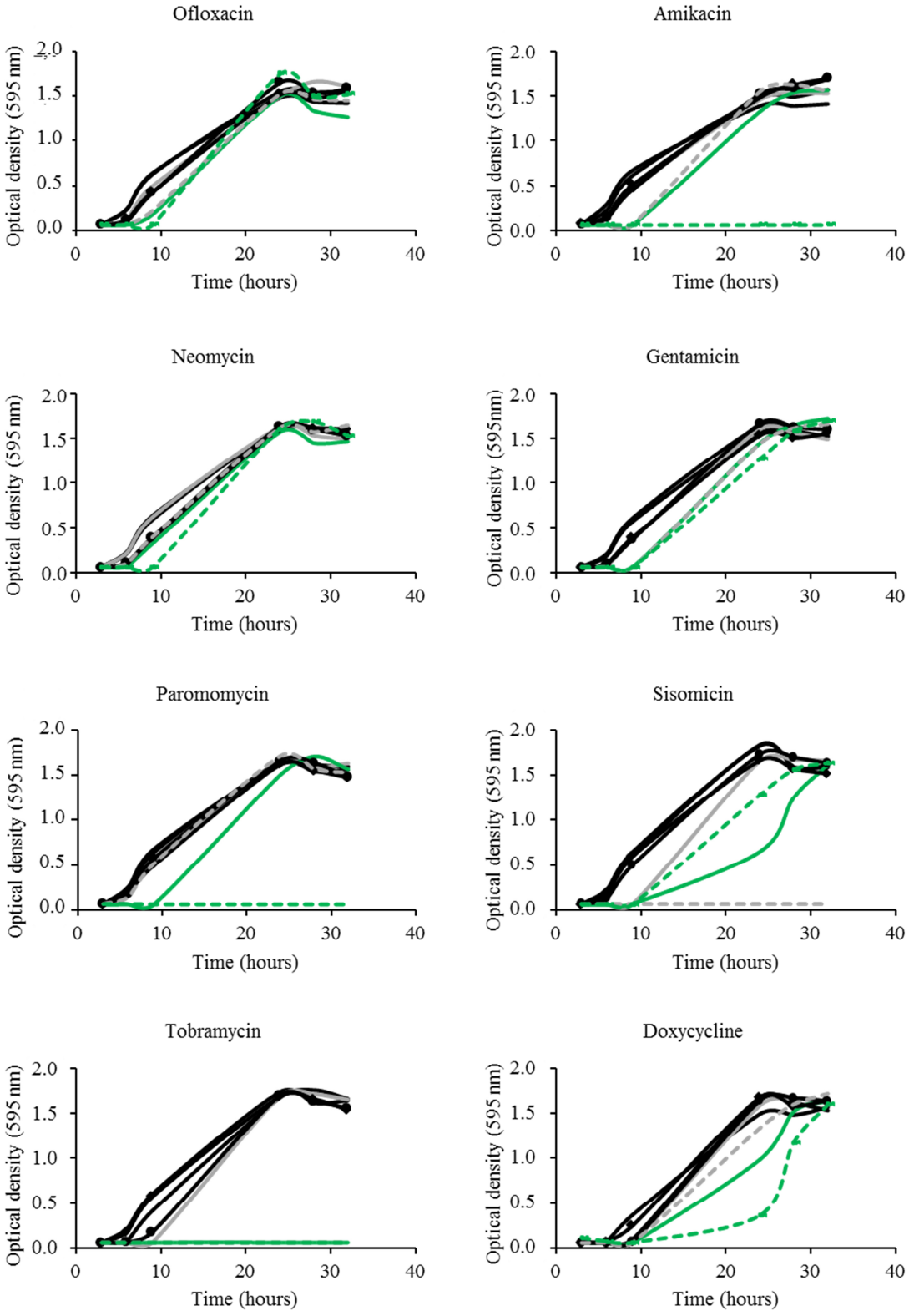
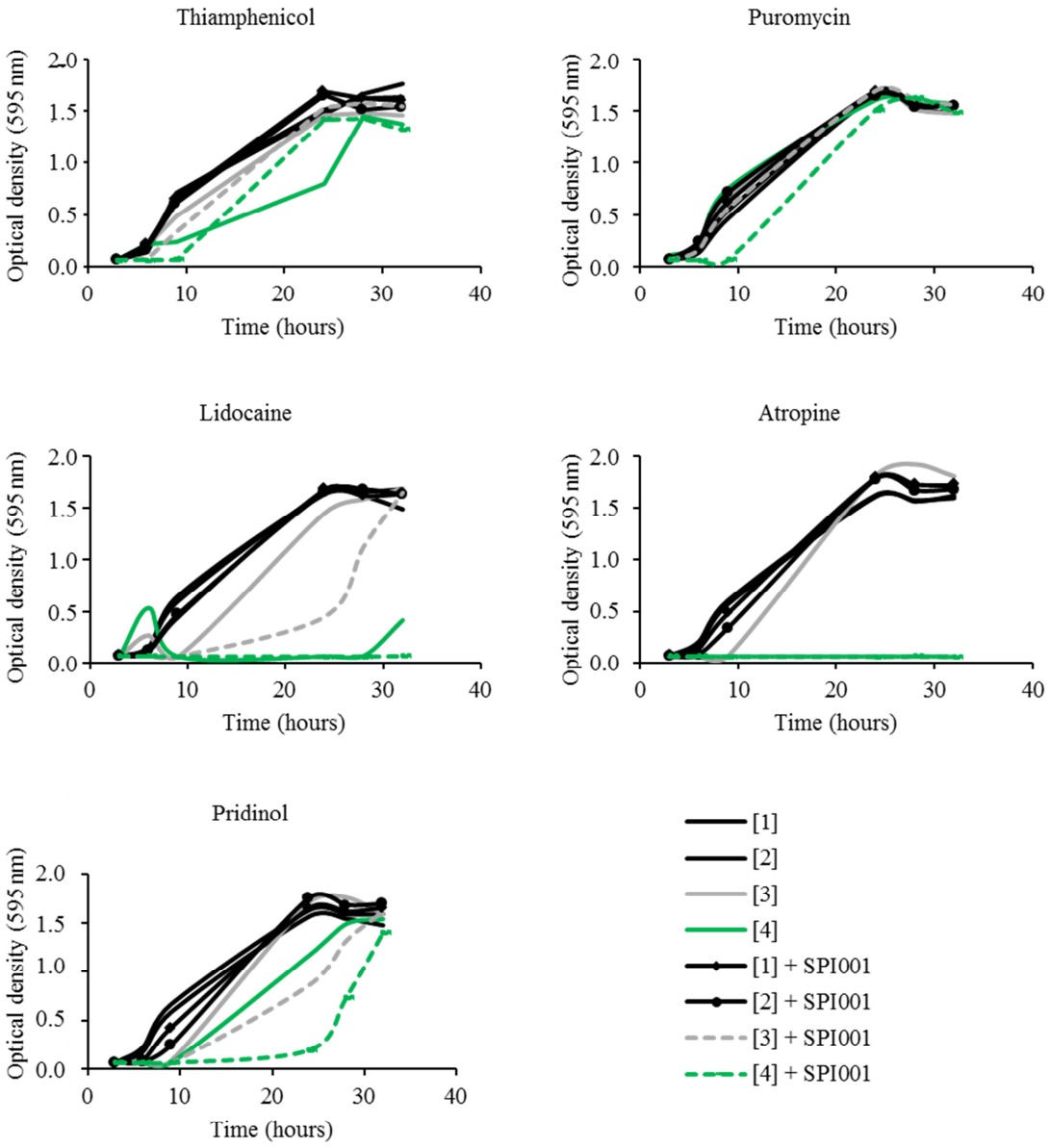


Figure B.4: Effect of SPI001 in combination with selected Biolog compounds (continued)



Continued Figure B.4: Effect of SPI001 in combination with selected Biolog compounds. Stationary phase cells of YM64 were treated overnight with a combination of 50 µM SPI001 and four different concentrations of Biolog compound (depicted by [1]-[4]). After treatment, cells were 100-fold diluted into fresh growth medium and regrowth was monitored by measuring optical density at fixed time points.

List of publications

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Knapen, W. J., **Liebens, V.**, De Groote, V., Verstraeten, N., Kint, C., Jans, A., Fauvart, M., Michiels, J.(2013).Cell surface associated antibiotic tolerance in the pathogen *Pseudomonas aeruginosa*. Annual meeting organized by the Belgian Society for Microbiology and National committee for Microbiology.

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***FEMS Young Scientist Meeting Grant**

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***Oral presentation**

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